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Toxic Phosphorus Esters

Chemistry, Metabolism, and Biological Effects

Toxic Phosphorus Esters

Chemistry, Metabolism, and Biological Effects

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1960

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Toxic phosphorus..

PRINTE F AMERICA

This book is dedicated to

Dr. E. Y. SPENCER

in gratitude for nine years
of friendship and guidance

Preface

The organophosphorus compounds have drawn the interest of warfare scientists, agriculturists, physiologists, and biochemists. Practically they may serve as nerve gases for humans or as safe yet potent pesticides; academically their specificity has made them tools for studying such problems as nerve function and the nature of enzyme active centers.

This book is intended to serve two functions: to be a source book on organophosphate research and to show how our understanding of the events that follow organophosphate poisoning of animals can be understood in terms of events at the molecular level. The first function would be fulfilled simply by a large-scale tabulation of the tremendous number of studies conducted in this area. The second has meant that an attempt had to be made to evaluate these studies, to weigh any conflicting evidence, and to draw conclusions from an over-all survey of them. Even more important, I have attempted to show by the organization of the material how to a great extent our knowledge of the chemical properties leads us to understand the biochemical properties, how these in turn enable us to interpret events at the physiological level, on isolated or partially isolated tissues, and then how this grand complex of understanding enables us to comprehend what happens when organophosphates are applied to the whole animal. Finally I have tried to show how we can even now make our first tentative attempts at using this whole corpus of learning to design new selectively toxic organophosphates with predictable properties.

This scheme may seem grandiose, and in fact our knowledge in many essential areas is still painfully inadequate. Nevertheless it is my belief that the skeleton of this complete understanding already exists, and that by assembling this skeleton one may better see where our deficiencies lie.

The book is addressed equally to chemists and biologists: both will doubtless find places where explanations are spelled out perhaps in more detail than one or the other may think necessary. But there will be other places that may be obscure; these places will mirror the difficulties involved in drawing conclusions from the available evidence. I have tried hard to give briefly where possible the essence of the various topics, for the sake of those who are unfamiliar with these compounds and want a broad sketch; but I have in most cases gone on to a detailed scrutiny of the evidence so that the specialist may be able to evaluate my conclusions and draw his own.

I like to think of this book as a summary of the first phase of organophosphate research, a phase in which enough knowledge has been gained to permit a more sophisticated approach to the whole question of organophosphate action. I know of no better expression of this attitude toward scientific literature than that written nearly a century ago by the great physiologist Claude Bernard*:

"The spirit of man follows a necessary and logical course in the search for scientific truth. It observes facts, compares them, deduces appropriate results which it controls by experiment, to rise to more and more general propositions and truths. In this advancing labour, a man of science must, of course, know and deal with his predecessor's work. But he must be thoroughly convinced that this work is merely a support from which to go further. . . . Useful scientific literature, then, is pre-eminently the scientific literature of modern work which enables us to keep up with scientific progress; and even this must not be carried too far, lest it dry up the mind and stifle invention and scientific originality."

London, Ontario R. D. O'Brien
July 1960

Acknowledgments

In an attempt to overcome some of the inevitable shortcomings of a book written by one author covering many fields, I asked a number of friends and colleagues who are experts in various aspects to criticize and amend my first version of this book. Their response was splendid, and has been of inestimable help. I am proud to acknowledge their assistance. They are Drs. K. van Asperen, J. E. Casida, E. H. Colhoun, H. Martin, L. A. Mounter, E. Y. Spencer, E. H. Smith, and L. S. Wolfe.

During the preparation I have been greatly helped by talks about neurophysiology with Dr. E. H. Colhoun, about techniques with Drs. W. C. Dauterman and E. Y. Spencer, about p*K* values with Dr. N. E. Good, about radioactivity with Dr. J. R. Robinson, and about infrared spectroscopy with Mr. R. W. White.

The arduous task of converting illegible and untidy longhand into neat typescript was generously undertaken by Miss Sharon Lewis, Mrs. Lois Simpson, Mrs. Ann Marie George, and Miss Glenda Greer.

This book would not have been written but for the kind invitation of the author by Drs. T. C. Allen and J. E. Casida to spend a year at the University of Wisconsin in 1958–1959 as Visiting Associate Professor.

* Claude Bernard, "An Introduction to the Study of Experimental Medicine," 1865 (translated by H. C. Greene), p. 145. Henry Schuman, New York, 1949.

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CHAPTER 1

Introduction

Plan of this Book

This book will attempt to give a fairly complete account of the biochemistry and physiological properties of organophosphates, along with a sufficient account of their chemical properties to explain the biochemistry in chemical terms.

The concept underlying the whole of this work is that which Sir Rudolph Peters[32] called in 1931 "the biochemical lesion": the idea that the effects of many poisons and vitamin deficiencies may be explained in terms of interference with one or more enzyme systems, and that the gross physiological picture that results may be traced entirely to such a lesion. Although in the case of poisons he first used it in accounting for the properties of arsenicals and fluorine-containing compounds, our knowledge of organophosphates is now much more detailed than that of those other agents, and has perhaps even more thoroughly vindicated the original idea.

The story of the action of organophosphates is by no means complete, and debate is particularly vigorous on the problem of their mode of action in insects. Most mammalian physiologists, however, accept the fact that in acute poisoning the action of organophosphates can be almost exclusively attributed to inhibition of the cholinesterase of the nervous system. In the course of this book this generalization will be hedged about with provisos, exceptions, and cautious deferments of judgement (as is proper), but as a preliminary statement, it is a good generalization.

The chapter sequence is planned in an attempt to develop logically the idea that the toxic effects of the organophosphates are attributable to esterase inhibition, and that the esterase inhibition can be accounted for to a great extent by the chemical properties of the compounds. The extreme selectivity of some organophosphates for some esterases remain, however, a complete mystery. We do have a fairly good working knowledge of the mechanisms by which the compounds are metabolized in plants and animals, and these mechanisms are by no means of secondary importance. Many of the selectively lethal effects are to be attributed to variations in the pathways or extent of metabolism.

The nonenzymic properties considered in Chapter 2 are those which may be of either theoretical or experimental importance to those who work with these compounds. On the practical side, it is essential to know something of their stability, and what liberties may be taken with their storage and use. This is of crucial importance with very many of those which contain sulfur; much of the early work was made quite invalid by subsequent demonstration of the ease with which such internal rearrangements as isomerization and transalkylation occur. Such rearrangements may occur not only in preparation (particularly during distillation) but in storage, and commonly lead to drastic changes in the biochemical properties.

On the theoretical side, Chapter 2 should prepare the way for the next chapter, which describes the reaction with cholinesterase *in vitro*. Chapter 4 is concerned with what are later called "modifying reactions," that is, metabolic alterations of the organophosphate, some of which make them more reactive to esterases ("activating reactions") and others which reduce their reactivity.

More complexities are introduced in Chapter 5, which deals with a more physiological level of events, that is, the effects which organophosphates produce when applied to isolated whole tissues, particularly nerve and muscle. A full knowledge of this aspect (which we do not have) would assist greatly in constructing the picture of what happens when whole animals are poisoned by these compounds.

By this stage the individual pieces of the jigsaw puzzle have been examined, and it remains to put them together, a feat attempted in chapters 6 and 7. In the case of mammals and insects, an account is first given of what the effects of poisoning are. Then the knotty problem (which may perhaps never be answered with absolute certainty) of the causal chain of events leading to death is examined. The modifying reactions and their interactions *in vivo* are next considered, and it is hoped to give some idea of the enormous importance these reactions have in deciding whether a poisoned animal will live or die. We shall also discuss how a knowledge of these modifying reactions can account for such serious problems as synergism, when two compounds in doses which are innocuous separately may together cause death; and the resistance of insects (that bogy of the chlorinated-hydrocarbon insecticide story) which of recent years has become gradually more prominent.

In Chapter 8 the role of the plant is considered. Very often the plant is used as a meeting place for insect and insecticide, and frequently it is not a neutral in the battle. Some account of systemic activity is given, whereby the plant transfers some organophosphates from one part to another; this may be simply good economically (giving a good distribution of roughly sprayed insecticide), it may be a factor in selectivity, it may allow getting at an otherwise concealed pest. The problem of possibly hazardous residues on treated plants is also treated.

Chapter 9 ought to be the most interesting, and concerns the provoking and fascinating problem of selectivity. Here, once more an attempt is made to tie together some of the facts previously presented in order to show how we may account for some of the striking known cases of selectivity, such as the killing of internal insect parasites in farm animals by dosing the animal with certain organophosphates. More important still, it is hoped to work out some principles to be used in the future development of selectively toxic chemicals with predictable toxicity. The acid test of the usefulness of all the elaborate studies on organophosphates is, can we use them to invent new compounds without the tedious wasteful method of large-scale, semi-random synthesis and screening which has so far provided us with every synthetic insecticide in commercial use today?

Chapter 10 is a severely practical section. Doubts as to whether it should even have been included have been submerged by the reflection that an understanding of the techniques which have been developed in this field is as crucial as knowledge of the results. This is equally true if one is reading and evaluating the work of others, or is carrying out work himself. I have always found it particularly irritating to read of some elaborate analytical process (for instance), when I have not known why this particular process was used, why some steps were included, and what would happen if I modified it in an attempt to get an answer to a simpler problem than the writer was tackling.

The first appendix, on electronic interpretations, may be an offence to chemists, for whom it is (*a*) all too obvious and (*b*) not very professionally written. But I am convinced that future rational developments will be based upon extensive use of such fundamentals, and I have therefore leaned fairly heavily upon such ideas in some places in the text. Since I hope that scientists other than chemists may read this book, it seemed only fair to include a brief account of a topic which, though complex in full, can be used simply in many cases.

The second appendix is necessary because I have used in the text only the most familiar names for organophosphates, and the full chemical name only where no other is available. A glance at the compounds listed in this appendix gives an idea of the astonishing number and variety of organophosphates which have been made and studied.

History

The first esterification of alcohols and phosphoric acids is attributed to Lassaigne in 1820.[38] In 1903 and 1915, Michaelis published long papers dealing with the synthesis of compounds containing phosphorus and nitrogen, particularly amidates of phosphinic, phosphoric, and phosphorothionic acids.[45, 46] In 1906, Arbusow reported the synthesis of a number of phosphates and phosphonates which were forerunners of the compounds used as anticholinesterases today.[8] Esters of pyrophosphoric acid were made in 1914 by Balareff[10] and in 1930 by Nylen.[49] In 1932, Lange and von Krueger prepared some dialkyl phosphorofluoridates.[39]

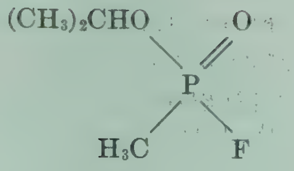
In all the early studies one finds no mention of the poisonous nature of these compounds. Some time in the 1930's it must have become apparent, for an intensive study of them as potential chemical warfare agents began in England and Germany in the early days of the last war.

The work in England was carried out by a group directed by B. C. Saunders at Cambridge. The compound that most attracted their attention was DFP, which they made in 1941, and the majority of other compounds they investigated also contained fluorine. It is rather alarming, in view of the extreme precautions practiced today, to note that the compounds were evaluated "(*a*) on ourselves, (*b*) on animals, and (*c*) on enzyme systems."[50] There has been a long gap in the human experimental studies since the war, and the recent ones were carried out under very different conditions of caution.

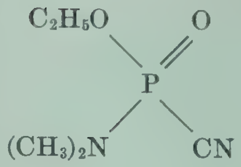
The properties that first attracted the attention of the English group were the high toxicity and the miotic effects (i.e., contraction of the pupil of the eye). Usually the compounds were applied as mists, with concentrations in the order of 1 part per million, and as a result they came to be known as "nerve gases." A considerable volume of work was produced on the relationship between structure and toxicity, which has been reviewed quite extensively by Saunders.[50]

The work in Germany was headed by Gerhard Schrader, of the I. G. Farben industries group. These workers had been investigating organophosphates since 1934, and their first patent (which was kept secret) was in 1937. They realized the insecticidal importance of these compounds, and by the end of the war had made many of the insecticidal phosphates in use today. Thus, dimefox was made by them in 1940, schradan (as it is now called, after its discoverer) in 1942, and parathion in 1944. The biological aspects of these compounds were studied by Schrader's colleague, Kükenthal. These workers also discovered the capacity of certain compounds (schradan in particular) to be translocated in the plant following root or leaf application; a property to which Martin and Shaw later applied the term "systemic activity,"[42] by analogy with similar familiar usage in medical terminology (Schrader had originally used the term "chemotherapeutic effect"[42]).

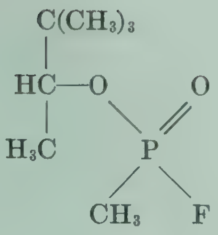
As well as these agriculturally useful compounds, the German workers made three compounds of extreme toxicity to mammals. They contained either F or CN substituents:



Sarin



Tabun



Soman

According to Saunders,[50] tabun was put into production at the end of the war in Germany, and a plant capable of producing 100 tons a month was built. Two plants for sarin production were being built at the end of the war. All three of these compounds have toxicities to mammals of less than 1 mg. per kg.

At the end of the war, all the German industrial and research secrets were made available to the Allied powers. The most significant documents were those recording the results of Schrader's work, which were obtained in 1946 by a British Intelligence Objectives Subcommittee (B.I.O.S.) under Hubert Martin, then at Long Ashton Research Station. The results were published in 1947 in the now famous B.I.O.S. Report No. 1095, followed soon after by Schrader's own account in B.I.O.S. Report No. 714. These reports contain an astonishing number of compounds, some of whose potentialities have only been realized in recent years.

The great importance of systemic action was fully realized by Martin and his colleague Bennett, and a number of studies were carried out at Long Ashton and elsewhere.[11] Nowadays systemic effects are recognized to be of great importance, and especially with the widespread use (particularly in the U.S.A.) of Systox and its relatives.

The first organophosphate insecticide in wide use was put on the market in Germany under the name "Bladan." It was originally considered to be hexaethyl tetraphosphate, but later studies showed that the active component was TEPP. Bladan was officially approved in Germany in 1944, and marketed as a substitute for nicotine against aphids.

The anticholinesterase property of organophosphates was first found in 1941 by a British group: Adrian, Feldberg, and Kilby, who published their findings in 1947.[2] The work originated with attempts to explain the pupil-constricting effects of DFP, which they found were purely local (in the case of vapor treatment). They studied various smooth-muscle-contracting compounds, and found, using a pharmacological preparation, that DFP behaved like eserine, in that it produced a contraction slowly and progressively, and the contraction was not readily reversed by washing the preparation. These observations led them to examine the anticholinesterase action of DFP (since eserine had long been known as a potent anticholinesterase) and they found that it was even more potent in this respect than eserine.

At about the same time, another Cambridge group, Mackworth and Webb,[41] carried out studies on the anticholinesterase activity of DFP, which showed that (unlike eserine) DFP inhibition was progressive and reversible.

In 1951, Diggle and Gage[19, 20] pointed out that the anticholinesterase activity of impure samples of parathion was almost entirely attributable to contamination by the *S*-ethyl isomer. This important observation was the first indication of the great difficulties, so characteristic of phosphorothionate chemistry and biochemistry, which later workers abundantly established.

The "activation" of phosphorothionates and phosphoramidates, i.e., the conversion of these weak anticholinesterases to potent inhibitors, was first suggested for the case of schradan in 1950 by Gardiner and Kilby[30] as a hypothesis, and later proved by them[31] to be caused by metabolism in the liver. A similar activation of parathion by liver slices was first shown by Diggle and Gage[21] in 1951; and Gage[29] showed in 1953 that the anticholinesterase produced *in vivo* was paraoxon.

The question of degradation of organophosphates in animals was first studied by Mazur in 1946, who found a so-called "DFP-ase" enzyme, which occurred principally in liver.[43] Since then, this and related systems have been studied in some detail, particularly by Mounter and Augustinsson. The increasing use of elaborate organophosphates with several alternative breakdown products has introduced new complexities into this field. The problem can be tackled only by chromatographic techniques: Metcalf's group in Riverside, California have used paper chromatograms successfully. Now column techniques, showing great promise for quantitative purposes, have been developed by Casida's group in Madison, Wisconsin. It is to be hoped that such methods will enable us to decide to what extent variations of susceptibility to organophosphates may be attributed to variations in degradation or activation of the compounds in the body.

In the postwar years, organophosphates have continued to be studied by defence research organizations all over the world, particularly at the Army Chemical Center, Maryland (in the U.S.A.), at Porton, Wiltshire (in England), and at Suffield, Alberta (in Canada). Naturally, these groups have been interested, mainly, in the compounds of high mammalian toxicity, and especially DFP, tabun, and sarin. For some years, these last two were secretly taken over from the German workers by the English-speaking groups. Recently there has been a considerable liberalizing of the attitude towards the basic research on organophosphates in defence groups, which has undoubtedly been for the good.

Recently there has also become available (mainly via *Chemical Abstracts*) an enormous amount of Russian work on organophosphates, mainly upon the chemistry and simple biological properties of the compounds.

Whether this liberalization is a consequence of the Western action, of political softening, or simply of the feeling that in an atomic age, organophosphates are mere toys, we can (as usual) only guess. It seems that U.S. interest in the uses of organophosphate in war has not vanished: a $50,000,000 plant for intermediates was built in 1953 in Muscle Shoals, Arkansas, to supply the Rocky Mountain Arsenal, Denver, Colorado, where final synthesis is accomplished. Production of war-gases was also continuing at Edgewood, Maryland in 1954.[7]

The last few years have been marked by the very great number of new insecticidal organophosphates that have been put on the market. The larger companies have been particularly desirous of obtaining compounds of low mammalian toxicity, partly for obvious reasons of extra safety and therefore extra sales, partly because the growing problem of resistance to chlorinated hydrocarbons (particularly DDT) suggests that if sufficiently safe and cheap organophosphates can be found they can be used on the gigantic scales hitherto reserved for chlorinated hydrocarbons. The most widely used of these new "safe" organophosphates have been Diazinon (of Geigy), malathion (of American Cyanamid), and Chlorthion (of Bayer).

For some years the attractive possibility has been studied that safe animal-systemic insecticides might be used to kill animal parasites. The chlorinated hydrocarbons have been extensively examined from this point of view, and with a little success. Much more dramatic effects have now been obtained with some new compounds, particularly Co-ral (of Chemagro and Bayer), and ronnel (of Dow). The control of cattle-grubs is the first target in most cases, but control of mites, mange, ticks, worms, and other parasites is also under study.

The spate of new insecticidal compounds is not yet abated. Since in the U.S.A. (and increasingly in other countries) the Federal authorities quite rightly demand extensive data upon the metabolism of new compounds in plants and animals, a great deal of such information has been obtained. Particularly productive in this area have been the groups with Metcalf at Riverside, California and with Casida at Madison, Wisconsin.

The requirement for ample data on the part of governments has so far been almost entirely beneficial scientifically. However, the spiralling costs of developing and registering a new insecticide in the U.S.A. (and no doubt in other countries) may lead in a few years to a phase of retrenchment. This probability is increased by the fact that by now the companies involved in the field are few and large, and may be prepared to accept a *modus vivendi* in which aggressive research and development is reduced all-around. Under such conditions, the insects may yet have the last word: if insects develop resistance to the established chemicals, the invention of new ones will be essential.

Nomenclature of Esterases and Organophosphates

Let us begin by defining an esterase as "any enzyme which hydrolyzes a carboxyester group." Now the field of esterase nomenclature is in a disordered state, mainly because most esterases are rather nonspecific. Thus, although we might (but shall not) define aromatic esterase as "an esterase having particularly high affinity for substrates in which the alcoholic substituent is phenolic," it would be quite incorrect to add phenyl acetate to a liver homogenate and ascribe the resulting hydrolysis to "aromatic esterase." Probably a little family of aromatic esterases would be at work along with other enzymes whose preferred substrates are radically different.

Quite apart from these erroneous usages, however, there is little agreement about the names to be applied to those enzymes whose individuality has been established. Some use the alternative name "lipase" if the substrate ester is a glyceride; some would apply the term "esterase" to enzymes cleaving noncarboxylic esters (e.g., phosphate esters). Commonly a prefix is added if a particular esterase has an affinity for one particular ester (e.g., cholesterol esterase), or is specific for one part of the ester (e.g., acetylesterase), or for one variety of ester (thus, aromatic esterases hydrolyze, mainly, aromatic esters; aliesterases hydrolyze aliphatic esters). An additional classification has been produced by Aldridge;[3] he uses the term "A-esterase" to describe esterases which cannot be inhibited by organophosphates, "B-esterase" for those that are inhibited.

In this book we shall try to avoid confusion by speaking of "hydrolysis of phenyl acetate," rather than using an enzyme name. Where this is cumbersome, the following imperfect definitions will hold: (*a*) cholinesterases are enzymes hydrolyzing acetylcholine; (*b*) aliesterases are enzymes which hydrolyze short-chain aliphatic carboxyesters with the exception of acetylcholine; (*c*) aromatic esterases are those which preferentially hydrolyze aromatic carboxyesters but have no action on acetylcholine; (*d*) phosphatases are enzymes which hydrolyze esters and thioesters of phosphorus acids; (*e*) amidases are enzymes which hydrolyze carboxyamide groups.

In the case of the enzymes that hydrolyze acetylcholine, only two are involved, and nomenclature ought therefore to be simple. It is not. The differentia for these two enzymes were authoritatively layed down by Augustinsson and Nachmansohn in 1949.[9] One of the enzymes we shall call here and throughout this book "acetylcholinesterase," the other will be called "pseudocholinesterase." Table 1.1 shows the differences between these two enzymes, and some of the alternative names that have been used. In this book we shall use cholinesterase (sometimes abbreviated to "ChE") as a nonspecific term to describe both enzymes. Unless otherwise stated, it can be taken that such a statement as "X inhibits cholinesterase" means X is effective against both enzymes. At times (usually when the

TABLE 1.1. DIFFERENCES BETWEEN ACETYLCHOLINESTERASE AND PSEUDOCHOLINESTERASE

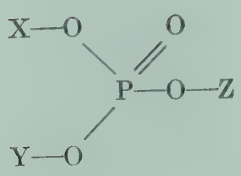
	Acetylcholinesterase	Pseudocholinesterase
Substrate most rapidly hydrolyzed	Acetylcholine	Propionyl and butyrylcholine
Will not hydrolyze	Most noncholine esters, butyrylcholine, benzoylcholine	Acetyl β-methylcholine
Excess substrate effect	Inhibited	Noninhibited
Main source	Erythrocytes, nervous tissue	Serum
Alternative names	Specific cholinesterase True cholinesterase *e*-Cholinesterase Acetylcholinesterase Acetocholinesterase Cholinesterase I	Nonspecific cholinesterase Pseudocholinesterase *s*-Cholinesterase Cholinesterase Cholinesterase II Serum cholinesterase

data under discussion demands it) the differentia used will be by source. Thus, we may speak of "red cell cholinesterase" or "brain cholinesterase." This has the disadvantage of not being very specific (for instance, brain has both acetylcholinesterase and pseudocholinesterase in it) but is very safe, because it is not going beyond the experimental facts: thus, "brain cholinesterase" can be safely used for total acetylcholine-hydrolyzing activity in a brain homogenate. Fortunately, choline esters are little hydrolyzed by enzymes other than the cholinesterase. But cholinesterases do hydrolyze a number of noncholine esters.[55]

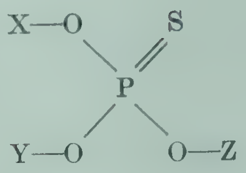
In this book, the term "organophosphates" is used loosely to apply to all compounds which contain carbon and are derivatives of a phosphorus acid. The terms "phosphate," "phosphorothionate," "phosphorothiolate," etc. will be used for more precise terminology, as described below.

The nomenclature of organophosphates has been in a confused condition, but in 1952 agreement was reached between Britain and the U.S.A. upon the naming of compounds containing one phosphorus atom.[6] Enough of the rules follow to make it clear how the commonest organophosphates should be named.

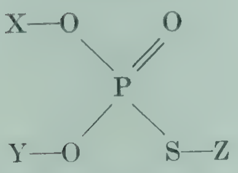
(*a*) The essential groups are as shown; underneath each is the latter half of the name to describe all derivatives of it.



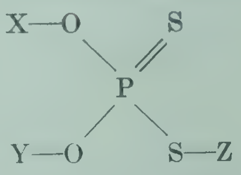
Phosphate



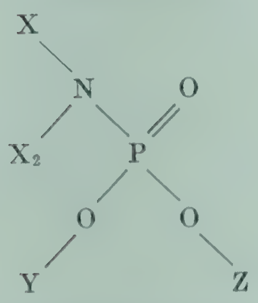
Phosphorothionate



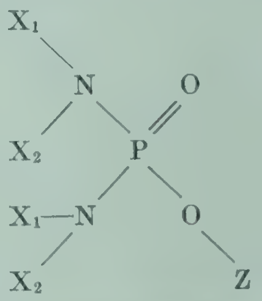
Phosphorothiolate



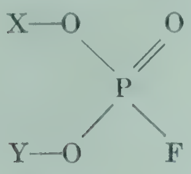
Phosphorodithioate



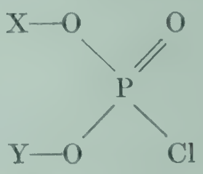
Phosphoramidate



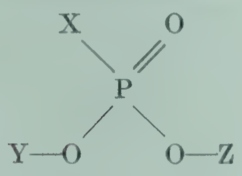
Phosphorodiamidate



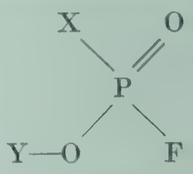
Phosphorofluoridate



Phosphorochloridate

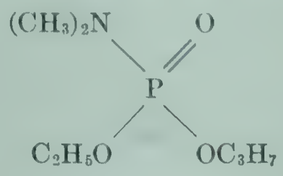


Phosphonate



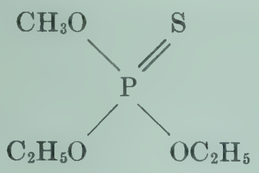
Phosphonofluoridate

(*b*) The substituents X, Y, or Z are included in the name with an indication of the atom to which they are attached, e.g.,



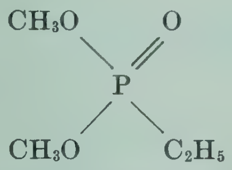
N,*N*-Dimethyl *O*-ethyl *O*-*n*-propyl phosphoramidate

But in those cases where there is no ambiguity, the atom of attachment can be omitted, e.g.,



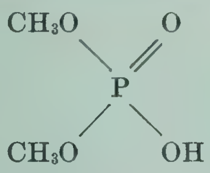
Methyl diethyl phosphorothionate

In the special case of compounds like phosphonates, containing a P—C bond, the convention is used of writing the name of the alkyl group which is attached to the phosphorus (i.e., by a P—C bond) in one word with the name for the acid part, e.g.,



Dimethyl ethylphosphonate

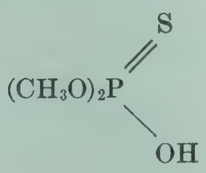
(*c*) If any of the above cases the X, Y, or Z of an OX, OY, or OZ group is hydrogen, the term "hydrogen" should be used in the name, e.g.,



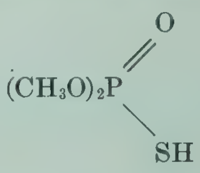
Dimethyl hydrogen phosphate

This rule is seldom observed, and will not be observed in this book. The above compound is normally called "dimethyl phosphate."

With regard to the suffixes "thionate" and "thiolate," some authors prefer to use "thioate" in place of both. In some cases this is judicious; for instance, there is debate about the state of the following isomers:

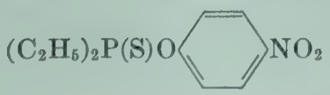


(I)



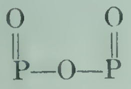
(II)

The first could be called dimethyl phosphorothionate; the second, *O,O*-dimethyl phosphorothiolate. But since it is uncertain which form predominates, the name *O,O*-dimethyl phosphorothioate may well be used, since it leaves the matter open. But compounds such as parathion



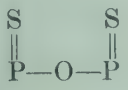
should be referred to as thionates rather than thioates, since there is no good reason to doubt whether the sulfur is as P=S or P—S—.

As for compounds containing two atoms of phosphorus, there is no international ruling; but by a sort of common consent, the following usage has been adopted. Most of the compounds are anhydrides having the P—O—P linkage. (*a*) If they are symmetrical about the P—O—P, call it: (X) phosphoric anhydride for



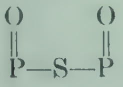
$$\overset{O}{\overset{\|}{P}}-O-\overset{O}{\overset{\|}{P}}$$

or (X) thionophosphoric anhydride for



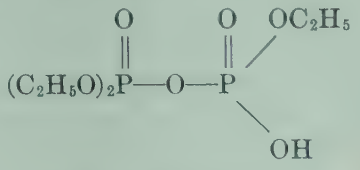
$$\overset{S}{\overset{\|}{P}}-O-\overset{S}{\overset{\|}{P}}$$

or (X) thiolophosphoric anhydride for



$$\overset{O}{\overset{\|}{P}}-S-\overset{O}{\overset{\|}{P}}$$

The name represented by X is the one that one would apply to the hydrolysis products of the molecule. Thus TEPP, $(C_2H_5O)_2P(O)OP(O)\cdot(OC_2H_5)_2$, is diethyl phosphoric anhydride. Schradan, $[(CH_3)_2N]_2P(O)\cdot OP(O)[N(CH_3)_2]_2$, is called bis-*N*-dimethyl phosphorodiamidic anhydride. (*b*) If the compound is unsymmetrical, use the old procedure of naming the compound as a substituted pyrophosphate.



Triethyl pyrophosphate

Unfortunately, these "transatlantic rules" are not accepted internationally. Larsson *et al.*[40] in 1954, drew up a table showing five different systems of nomenclature, two American, one British, one German, and the new Anglo-American. Unfortunately they proceeded to add a sixth, which was recommended by Sweden. Their objections to the Anglo-American system included those that it introduced new endings and was "too perfectionistic." Since then, the Swedish workers have kept firmly to their own scheme. As a result, the many excellent papers on the topic in *Acta Chemica Scandinavica* and *Acta Physiologica Scandinavica* have to be interpreted via the heretical code of Larsson.

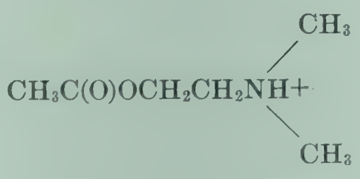
Structure of Cholinesterase

Cholinesterase is a celebrated enzyme in the biochemical world because such a great deal of information has been gathered about the nature of its active surface even though the enzyme has never been crystallized. Because this book is primarily about organophosphates, we shall not attempt to give a full account of how the current conceptions about the active surface were arrived at, except where there is some doubt about the correctness of some particular facet.

The bulk of our knowledge comes from a classic series of papers by I. B. Wilson and Felix Bergmann working at first together, and later separately with collaborators in New York and Jerusalem, respectively. Most of their work is on acetylcholinesterase derived from the electric eel, *Electrophorus electricus*, an animal which generates electricity in a special organ which is remarkably rich in cholinesterase.

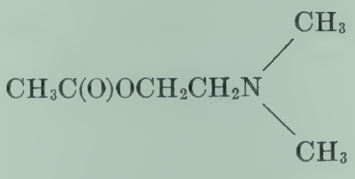
The primary conclusions are that cholinesterase has in its active center two kinds of site, one called the esteratic site, the other the anionic. The anionic site carries a negative charge, and binds the quaternary nitrogen of acetylcholine, thus increasing the specific affinity of the enzyme for the substrate. The esteratic site carries out the hydrolysis of the substrate, and also the hydrolysis of other less specific substrates; it is the site which organophosphates attack. The esteratic site contains an acidic group (whose neutralized form, or conjugate base, is inactive) and a basic group (whose protonated form, or conjugate acid, is inactive).

The evidence for the presence of an anionic site is: (1) The substrate diethylaminoethyl acetate, the tertiary analog of acetylcholine, is hydrolyzed about 3 times faster at pH 7, when it is mainly in the protonated form (I), then at pH 10, when it is mainly in the free base form (II) — this rate ratio is corrected for differences in enzymic activity at the two pH values.



$$CH_3C(O)OCH_2CH_2\overset{CH_3}{\underset{CH_3}{NH+}}$$

(I)



$$CH_3C(O)OCH_2CH_2\overset{CH_3}{\underset{CH_3}{N}}$$

(II)

(2) The inhibitor eserine, also a tertiary base, is relatively ineffective at high pH (free base form) and several times more effective at low pH (protonated form).

These two pieces of evidence[56] are only qualitatively good: there is very poor parallelism between the effectiveness of these compounds and the fraction ionized at a series of pH values. This may be ascribed partly to the fact that even the un-ionized forms have fairly substantial effectiveness and partly to the difficulty of correcting accurately for changes of the enzyme surface with pH. This latter difficulty is twofold. (1) Since both the inhibitor and substrate given above bind to the esteratic as well as to the anionic site, one has to correct for variations in the binding of the esteratic site with pH. For this correction the variation of acetylcholine hydrolysis with pH must be used, and this is not an entirely satisfactory correction. (2) The assumption is made that the structure of the anionic site does not vary with pH. As will be seen below this is not correct at all pH values which have been studied.

The evidence that there is an esteratic site separate from the anionic site is: (1) the ester group of the substrates (such as acetylcholine and dimethylaminoethyl acetate) is separated by 2 methylene groups from the cationic part of the molecule; (2) the effectiveness of un-ionized, un-ionizable inhibitors, which (since they lack a cationic structure) cannot be bound on the anionic site, varies markedly with pH. Thus TEPP exhibits a clear inhibitory maximum around pH 8 (see Fig. 3.4, Chapter 3). Since the inhibitor's structure cannot change with pH, this maximum must reflect changes in the enzyme site. Wilson and Bergmann[56] account for the maximum by assuming that it represents the superimposition of two pK curves of an acid and a basic group which are present within the site. If at pH values near 7 the acid is A and the base is BH^+, they suggest that only the combination ($A + BH^+$) binds the inhibitor. The optimum pH of such a site, i.e., the pH at which the concentration of ($A + BH^+$) is maximal, is the mean of the two pK_a values. At lower pH values, the limiting factor will be the amount of A as yet unprotonated; at higher pH, the limiting factor will be the amount of BH^+ still protonated. In Fig. 1.1 a plot is given of an acid of pK 6 and a base of pK 7, showing how much A and BH^+ is present at each pH. The solid line shows the concentration of ($A + BH^+$) at each pH, and should parallel the plot of enzyme activity (or, more precisely, esteratic site activity) against pH.

A precisely similar argument may be used to explain the optimum in the relationship between hydrolysis of acetylcholine and pH, as long as one may neglect the changes of the anionic site with pH. However, the maximum effectiveness of acetylcholine hydrolysis extends over a broad peak between pH 7.5 and 9 (this is true of acetylcholinesterase, not of serum cholinesterase) as shown in Fig. 1.2, and quite unlike the curve for TEPP inhibition (see Fig. 3.4, Chapter 3). This was suggested by Bergmann and Shimoni[14] to be due to the fact that the anionic site as well as the esteratic site was pH-sensitive in the range studied, since the major difference between the binding of the two compounds was that acetylcholine would be bound at both sites, TEPP only at the esteratic site. In order to

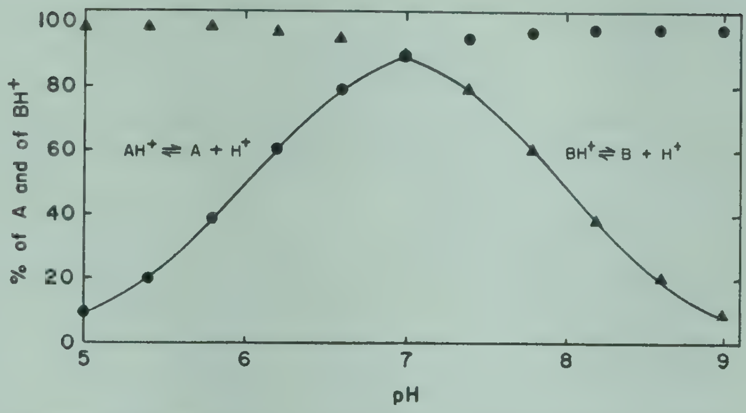


FIG. 1.1. Variation with pH of production of A from AH^+ (circles) and of BH^+ from B (triangles), when the acid AH^+ has a pK_a of 6.0, the base B a pK_a of 8.0. The line gives the amount of $(A + BH^+)$ as a function of pH; the amount is maximal at the mean pK_a .

test their suggestion, they studied four quaternary nitrogen inhibitors, of which two could not bind to the esteratic site, and found only small changes of inhibition with pH at about pH 7; however below that there was a sharp decline, so that at pH 5 inhibition was negligible (Fig. 1.3). Presumably these changes reflect alterations in the structure of the anionic site with pH. The authors suggested that the site might contain a carboxylate ion, whose ionized form COO^- was responsible for binding cations. The pK clearly lies between 5 and 7. Bergmann[12] estimates pK_a values of 5.75 for the anionic site of acetylcholinesterase and 6.2 for the serum enzyme, both of which are high for the nearest possible candidate carboxyl compounds (glutamic acid $pK = 4.25$ for γ-carboxyl; aspartic acid $pK = 3.65$ for β-carboxyl). It would seem, then, that above pH 7 variations in the anionic site cannot be evoked in explaining the variation of acetyl-

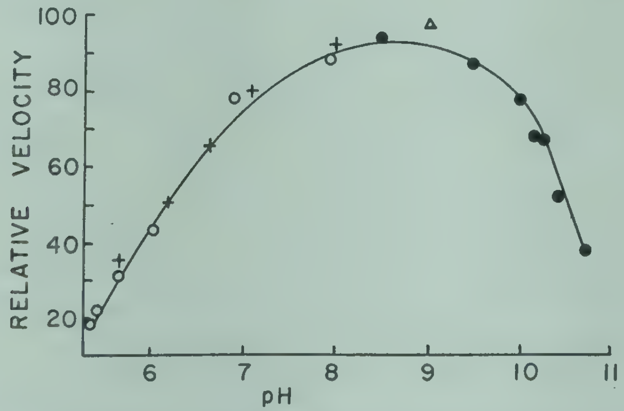


FIG. 1.2. Acetylcholine hydrolysis by purified electric eel acetylcholinesterase as a function of pH. From Wilson and Bergmann [*J. Biol. Chem.* **186,** 683 (1950)].

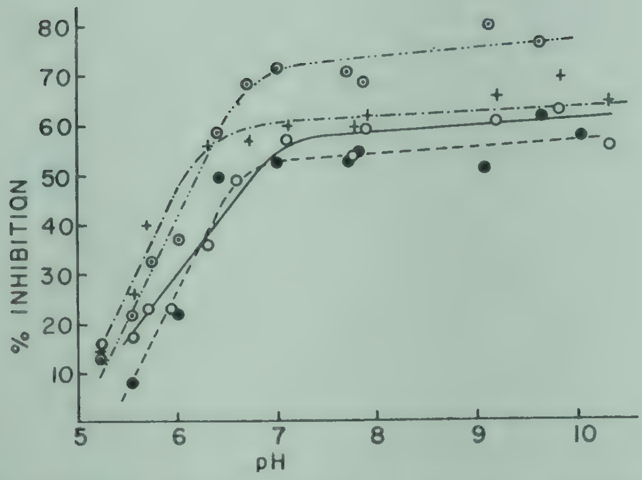


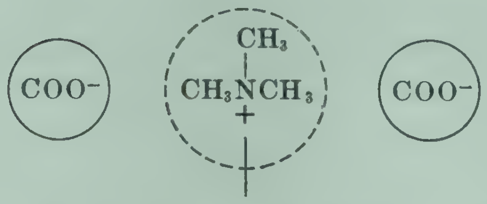
Fig. 1.3. Inhibition of purified electric eel acetylcholinesterase by quaternary ammonium salts as a function of pH. From Bergmann and Shimoni.[14]

⊙–··–··⊙ Choline chloride 10^{-2} *M*
+—·—·+ Tetraethylammonium bromide 5 × 10^{-3} *M*
○——○ Acetophenone *m*-trimethylammonium iodide 8 × 10^{-6} *M*
●----● Prostigmine bromide 5 × 10^{-3} *M*

choline hydrolysis with pH, and the broad maximum observed is still unexplained.

Two important modifications to the early theory have been introduced by Bergmann: (*a*) van der Waal's forces (short-range polarization forces) acting upon the intermediate carbon chain are important as well as the coulombic forces described above which bind the carbonyl and quaternary groups. Thus, in a series of *n*-alkyl trimethylammonium salts, the binding (as measured by inhibitory power) increased linearly with the length of the alkyl chain. Presumably, this binding is to parts of the enzyme other than the esteratic or anionic sites. Bergmann and Segal[13] calculate that the energy derived from binding each methylene group is about 0.5 kcal. mole^{-1} for pseudocholinesterase and 0.3 kcal. mole^{-1} for acetylcholinesterase. (*b*) Pseudocholinesterase has one anionic site for each esteratic site; acetylcholinesterase has two. This conclusion[15] follows from evidence that the serum enzyme does have an anionic site (as judged by the pH dependence of inhibition by compounds which would bind to an anionic site) taken with the calculation of Adams and Whittaker[1] that acetylcholinesterase had one more site than the serum enzyme, based on differences in binding of choline to the two enzymes.

Bergmann[12] suggests that the two anionic sites of acetylcholinesterase bind as follows (the circles give a rough picture of the relative sizes, assuming that the anionic sites are carboxylic):



We now come to the problem of the precise structure of the esteratic site. The approach here has been by an analysis of the pH dependence of hydrolysis or inhibition.[12] With all neutral substrates, the shape of the pH activity is very similar. The principles already described are formulated as follows. As given above, the hypothesis is that AH^+ and B are active forms, A and BH^+ are not, i.e., AH^+B is active, but AH^+BH^+ or AB are not:

$$\underset{\begin{pmatrix}\text{inactive}\\ \text{low pH}\end{pmatrix}}{AH^+BH^+} \underset{K_a}{\rightleftharpoons} \underset{(\text{active})}{AH^+B} \underset{K_b}{\rightleftharpoons} \underset{\begin{pmatrix}\text{inactive}\\ \text{high pH}\end{pmatrix}}{AB} \tag{1}$$

Writing E for the composite site AB, then EH^+ is the active form. The equilibria K_a and K_b in (1) are those of the ionization of A and B, as shown in Fig. 1.1. The two ionizations are described by:

$$K_a = \frac{(EH^+)(H^+)}{(EH_2{}^+)} \tag{2}$$

$$K_b = \frac{(E)(H^+)}{(EH^+)} \tag{3}$$

Next, we consider the variation of activity with substrate concentration and utilize the fact that inhibition is produced by an excess of substrate. This observation is formulated according to the Haldane treatment whereby the inhibition is considered to be due to addition of substrate to some of the enzyme-substrate complex to produce an inactive complex:

$$EH + S \underset{K_1}{\rightleftharpoons} EHS \rightarrow \text{products}$$

$$EHS + S \underset{K_2}{\rightleftharpoons} EHS_2 \ (\text{inactive})$$

From these equilibria:

$$K_1 = \frac{(EH)(S)}{(EHS)} \tag{4}$$

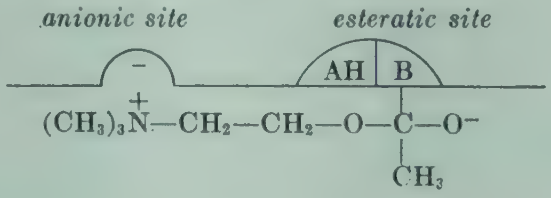
$$K_2 = \frac{(EHS)(S)}{(EHS_2)} \tag{5}$$

Equations 2 to 5 may now be used to calculate K_a and K_b, the dissociation constants of the acid and basic groups of the esteratic site. For a number of esterases and un-ionized substrates (which are not complicated by anionic site effects) the pK_a values of the acid and base are about 6.5 and 9.2 respectively. The acid of pK 6.5 may be the conjugate acid of histidine (see pages 81–83).

Having considered the parts of the enzyme item by item, let us see how the whole active center attacks acetylcholine. The picture to be presented is essentially that of Wilson *et al.*,[57] and some of the details are as yet unproven. Only one anionic site will be shown, but it is understood that two are involved for acetylcholinesterase.

In stage 1, the cationic part of the substrate is coulombically bound to the anionic site, and the carbon of the ester group (which is electrophilic) is coulombically bound to the nucleophilic basic group B of the esteratic site, with which it then forms a covalent bond (*a*):

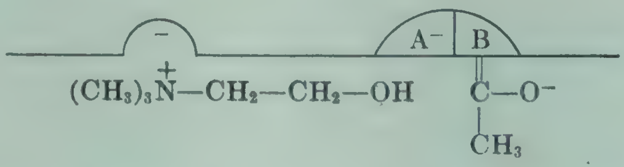
(*a*)



$$(CH_3)_3\overset{+}{N}-CH_2-CH_2-O-\underset{CH_3}{C}-O^-$$

This combination is the rate-controlling stage of the reaction. A hydrogen bond then forms between AH and the O of the C—O—C, followed by a rearrangement with cleavage of the O—C bond and a removal of hydrogen from AH to the choline moiety (this is the over-all effect; there is no proof that the particular H is transferred) (*b*):

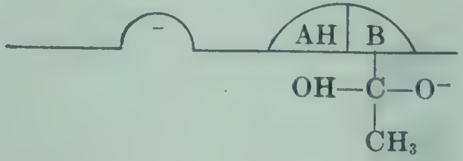
(*b*)



$$(CH_3)_3\overset{+}{N}-CH_2-CH_2-OH \qquad \underset{CH_3}{C}-O^-$$

An attack is now made by a water molecule on this acetylated enzyme. The group A recovers a proton and the acetyl group picks up a hydroxyl, while the choline molecule diffuses away, and one may picture the highly transient condition (*c*):

(*c*)



$$OH-\underset{CH_3}{C}-O^-$$

The BC bond promptly breaks, restoring the original condition of the site, and producing acetic acid which immediately ionizes.

A series of papers by Friess and associates (e.g. refs. 24, 26, 27) has confirmed the essence of the two-site theory, but suggests that the esteratic site binds to a negative group of the ester, rather than to the positive carbonyl carbon. They found that the requirement for strong binding of an ester to acetylcholinesterase was a tertiary or quaternary nitrogen separated by a $—CH_2—CH_2—$ group's distance from a region of high electron density, whereas the carbonyl carbon is a center of low electron density, due to electron withdrawal by $=O$. Thus β-chlorocholine

$$(CH_3)_3N^+CH_2CH_2Cl$$

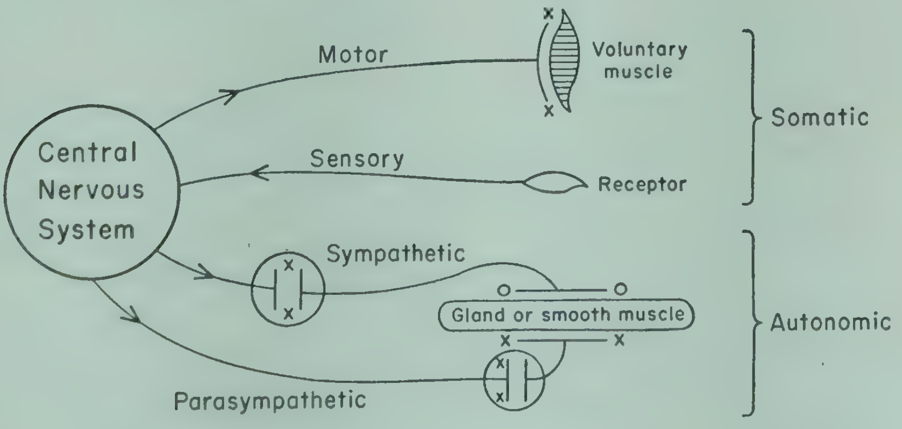
proved to be an extremely potent anticholinesterase, whereas choline was an extremely poor one. Clearly the Cl binds strongly to the esteratic site. This observation suggests that acetylcholine may be bound to the esteratic site at the electronegative carbonyl or esteratic oxygen rather than a carbon. Wilson has no evidence to offer on this point.

Further work by Friess and Baldridge[25] has resulted in the preparation of quaternary esters which are better substrates than acetylcholine, e.g., *cis-d-l*-trimethylaminocyclopentanol. Using *cis* and *trans* isomers of this compound, in which the quaternary and esteratic groups are separated rigidly (rather than flexibly, in the case of the two methylene groups of acetylcholine), they conclude that the maximal distance between the N and the esteratic O is about 2.5 angstroms.

Biological Significance of Cholinesterase

A brief picture of the structure and function of the nervous system will be necessary to explain the significance of cholinesterase. The mammalian system will be described first.

The nervous system has two major subdivisions: the central nervous system, (i.e., the brain and spinal cord) and the peripheral system. The peripheral system has two subdivisions, the somatic system and the autonomic system (see Diagram I).



(I)

Adrenergic = o——o
Cholinergic = x——x

(*1*) *The Somatic System.* This system is subdivided into two types, motor and sensory.

(*a*) The motor system is that by which efferent (outgoing) impulses are delivered from the central nervous system to voluntary muscles (e.g., of the leg or arm) and so induce appropriate movement in them. These voluntary muscles consist of all those muscles over which we have conscious control. Let us consider a single unit of the motor system: from a motor neuron in the central nervous system there emerges an axon, which is a fiber which can convey electric impulses. The axon terminates in the motor end-plate situated in the muscle: stimulation of the end-plate by an electric impulse delivered by the axon may cause the muscle to contract. However, there is no direct electrical contact between the axon and the muscle. One can picture it as if a small gap existed in the electrical circuit. This gap which is situated in the end-plate, represents the junction of the axon with the muscle itself, and is called the neuromuscular junction. When an appropriate impulse arrives at the junction, it releases a tiny "packet" of acetylcholine, which diffuses across the gap, and stimulates the muscle to contract. The system is said to be cholinergic, i.e., mediated by acetylcholine.

(*b*) The sensory system is that by which afferent (incoming) impulses are received from external receptors (e.g., in eye, ear, and skin) so that the brain is kept informed of the state of the outside world; and from internal receptors (e.g., in muscle and gut) so that the brain is kept informed of the status of the body—its position in space, the tonus of its muscles, and "deep pain." Receptors are usually specialized organs (e.g., the retina of the eye) or sometimes only diffuse fiber endings. In any case, there is probably continuity between the potential-generating part of the receptor and the axon of the sensory nerve, so there is no gap comparable with the neuromuscular junction. The axon leads (without synapsing) from the receptor to the central nervous system.

(*2*) *The Autonomic System.* This system supplies nerves to (innervates) the muscle systems which are other than voluntary [i.e., heart, smooth muscle (which is the muscle in the alimentary canal), eye pupil, bladder, etc.], and the glands. It has two subsystems which work contrarily: the sympathetic and the parasympathetic. If one subsystem stimulates an organ one way, the other often stimulates it in the opposite way. (Some organs, however, are only innervated by one subsystem, e.g., the lacrimal glands have only parasympathetic innervation.) Some examples are given in the tabulation.

Organ	Sympathetic stimulus	Parasympathetic stimulus
Pupil	Dilates	Contracts
Lacrimal gland	—	Causes secretion
Salivary gland	Causes sparse mucinous secretion	Causes profuse watery secretion
Bronchioles	Dilates	Constricts
Alimentary canal	Decreases motility and tone	Increases motility and tone
Heart	Accelerates	Retards
Sphincter of bladder	Contracts	Relaxes

(*a*) The sympathetic system is one in which there is no direct axonic connection between the central nervous system and the organ (with the single exception of the supply to the adrenal gland). Instead, the axon from the central nervous system is interrupted by a so-called synapse, another gap in direct electrical continuity. The synapses of the sympathetic system are gathered together in ganglia, of which a familiar example is the solar plexus. The synaptic gap is bridged by acetylcholine, just as is the neuromuscular junction. In this case, acetylcholine is released when an impulse travelling along the axon between the central nervous system and the ganglion (the preganglionic axon) reaches the synapse. The acetylcholine then stimulates a fresh impulse in the axon which joins the synapse to the organ (the postganglionic axon). Once more there is a "gap" between the postganglionic axon and the organ; but in this case, the "gap" is bridged not by the release of acetylcholine, but by the release of adrenaline or noradrenaline. Such a system is said to be adrenergic.

There are two exceptions to the rule that sympathetic innervation of organs is adrenergic; i.e., the innervation of the adrenal medulla and sweat glands is cholinergic.

(*b*) The parasympathetic system, anatomically, is somewhat like the sympathetic. But its synapses outside the central nervous system are not all gathered up into a chain of relatively large ganglia; some indeed are in large ganglia (e.g., the ciliary and stellate ganglia) but the majority have their synapses in small ganglia lying near their effector organs, so that the postganglionic axon is commonly very short. Physiologically, the parasympathetic system differs from the sympathetic in that both its pre- and its postganglionic nerves are cholinergic.

All of the three systems described originate in the central nervous system, in which they have further synapses. Little is known about the mediator of the central synapses (for some synapses, serotonin or 5-hydroxytryptamine has been suggested, and for others, γ-aminobutyric acid) but since many of the symptoms of poisoning by anticholinesterases are often central it is probable that at least some central mediation is cholinergic.

Let us now consider any of the cholinergic systems, i.e., the neuromuscular junction, the parasympathetic neuroeffectors, the sympathetic synapses, and the sympathetic innervation of the adrenal and sweat glands.

When the "packet" of acetylcholine is released by the incoming stimulus, it diffuses across the "gap" and stimulates either an organ effect or (in the synapse) initiates events leading to a fresh impulse. If the acetylcholine were allowed to persist, it would continue its stimulation and destroy the required proportionality between input stimulus and event. In practice, the acetylcholine is destroyed very promptly by cholinesterase, the enzyme which is present in a large excess. If the cholinesterase should be prevented from acting (e.g., by inhibition by an anticholinesterase), acetylcholine will accumulate, causing at first an excessive stimulation, and finally complete disruption (or block) of the cholinergic system.

The result of inhibiting cholinesterase will thus be (*a*) interference with the neuromuscular junction, giving rise to fasciculation (rapid twitching) of voluntary muscles, and finally paralysis, which is of particular importance in the respiratory system; (*b*) interference with the autonomic nervous system at the cholinergic sites; in general, the symptoms seen are those caused by excessive parasympathetic stimulation: pupil contraction, secretion of tears and saliva, and constriction of the bronchioles. Central effects may also occur, such as incoordination and paralysis of the respiratory center.

Four compounds of pharmacological importance in relation to cholinergic systems are: nicotine, muscarine, atropine, and curare. These compounds are not normally present in the body.

Nicotine acts as acetylcholine does, but only at the neuromuscular junction and at the ganglion. It therefore gives rise to stimulation of voluntary muscles, and of the sympathetic innervation of smooth muscle and glands. Those symptoms caused by applied acetylcholine or anticholinesterases which resemble those in nicotine poisoning are called "nicotinic," e.g., paralysis of respiratory muscles, fasciculation.

Muscarine acts as acetylcholine does, but only at the junction of the parasympathetic axon with the organ. Those symptoms caused by applied acetylcholine or anticholinesterases which resemble those in muscarine poisoning are called "muscarinic," e.g., slowing of the heart, constriction of the pupils, urination, and salivation.

Atropine antagonizes acetylcholine action, but only the muscarinic actions and some central effects. Presumably, it does this by blocking the receptor site to which acetylcholine normally attaches itself, and so prevents its action. Atropine is poisonous by itself, but small amounts are effective against poisoning by acetylcholine or organophosphates, as described in detail below (Chapter 6). The arrow-poison curare (or its pure form, *d*-tubocurarine) antagonizes acetylcholine, but only at the neuromuscular junction.

The account given so far has made no mention of the mechanism by which electrical impulses travel along the axon. An excellent review of the mechanism has been given by Hodgkin,[35] who has been responsible for much of the elegant work which he describes. Only a few statements can be made here, enough to comment on the possible role of cholinesterase in axonic transmission. (*a*) Transmission is not passive (as when a current traverses a wire) but active: the nerve has to be in an excitable state, and metabolic work is done by the nerve to this end. (*b*) The resting nerve has a certain potential difference across its axon sheath. The membrane is positive on its outside and negative on its inside. The source of this potential is twofold: the sodium level outside the nerve is greater than on the inside, and the potassium level is less on the outside than on the inside. The low sodium level is maintained by a "sodium pump," a metabolic device whereby sodium is constantly removed from the inside to the outside of the nerve, thus overcoming the steady slow diffusion in from the outside. (*c*) The impulse that moves down a stimulated nerve is associated with a wave of reversed polarization. This wave is twofold: its first part is due to a rushing in of sodium in response to a transient "leakage" of the membrane; its second part is due to a transient "leakage" out of potassium.

The theory still leaves unanswered a crucial question: what causes the initial breakdown in the axonic membrane, allowing the sodium inrush, and how is this transient breakdown transmitted along the axon?

Nachmansohn and his colleagues at Columbia University have attempted to offer an answer to this problem.[16, 17, 47, 58] They feel that axonic transmission is inseparably associated with the presence of cholinesterase and acetylcholine, and that acetylcholine plays the role of transiently reducing the ion-permeability of the axonic membrane. The effect is transient because of the rapid destruction of the acetylcholine by cholinesterase. Some of the arguments in favor of this hypothesis are as follows:

(*a*) Cholinesterase is present in conductive tissue throughout the animal kingdom, and in adrenergic as well as cholinergic nerves.

(*b*) The enzyme has an unusually high speed of reaction, sufficient to account for the rapid changes that must be involved in conduction of nerve impulses.

(*c*) DFP blocks axonic conduction and concurrently inhibits cholinesterase. In nerves blocked irreversibly by DFP, cholinesterase is absent. As long as conduction is maintained, cholinesterase is always present (but only observed if sensitive techniques are used for its assay).

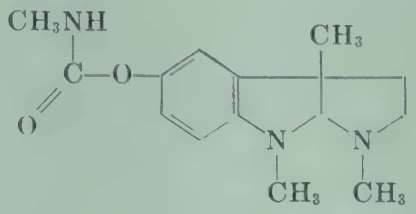
(*d*) Using intact (crab) nerves, one may distinguish between "external" and "internal" cholinesterase: the external hydrolyzes both acetylcholine and dimethylaminoethyl acetate, the internal hydrolyzes the latter. Those anticholinesterases (such as TEPP, prostigmine, and decamethonium) which do not produce conduction block, inhibit only external cholinesterase. Those (such as DFP and eserine) which do produce conduction block inhibit both external and internal enzyme.

(*e*) In the electroplax of the electric eel, there is a direct correlation between the amount of cholinesterase present and the voltage produced.[48]

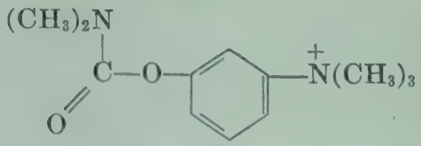
Some of the objections to Nachmansohn's views are:

(*a*) The methodology used is questionable.[37] The literature is full of argument as to whether cholinesterase is indeed present in (say) DFP-treated animals, whose axonal conduction may be unimpaired. Certainly, standard Warburg assays show that none remains. Nachmansohn's group object to the insensitivity of this assay and show that an alternative method, in fact, shows considerable residual enzyme. They also feel that the problem of retention of inhibitor in the tissue, and its later coming into contact with cholinesterase during homogenization, has not been sufficiently controlled by other workers.[17] Their own techniques have met with considerable criticism, however.[37] Only a detailed discussion would do justice to this dispute.

(*b*) Acetylcholine, in very large concentrations, has no effect upon axonic transmission.[23, 28] Nachmansohn has suggested that this is due to poor penetration of the axon by the cationic compound. In support of this, cationic compounds such as curare (a mixture of alkaloids) and prostigmine were shown to be ineffective against axonic transmission when applied to the axon, yet were very effective blocking agents when injected[33]; in fact, by injection, these compounds were more effective than such tertiary compounds as eserine and stilbamidine, which were effective by outside application too. (*Note:* prostigmine and eserine are anticholinesterases; stilbamidine is a curare-like compound.)



Eserine



Prostigmine

All this is convincing evidence that an ion-barrier protects the axon. However, even on injection into the axon, acetylcholine is a rather weak blocking agent; although 10^{-5} μg. of curare blocks conduction, 10^{-2} μg. of acetylcholine is needed for a similar effect. Such an effect is not convincing enough evidence.

(*c*) There is a sharp disagreement in results on the question of the presence or absence of certain enzymes. Nachmansohn's views require that all axons should contain acetylcholine, choline acetylase (the acetylcholine-synthesizing enzyme), and cholinesterase. In the optic nerve of the rabbit, Feldberg* states that choline acetylase is absent, but De Roetth found it present in substantial amounts,[18] in agreement with Nachmansohn.[47] Hebb[34] states that it is absent in optic nerves of cat, man, and ox.

(*d*) It has been shown[52] that although DFP blocks axonic conduction, it does not cause depolarization. In this way it resembles the action of local anaesthetics such as procaine. If it is true that it is acetylcholine which is responsible for the transient depolarization which occurs during nerve transmission, as Nachmansohn suggests, one would expect that a block of cholinesterase would lead to extensive depolarization.

Later work of Nachmansohn's group[5] with *Electrophorus* electroplax has confirmed the observation. Their extensive experiments show that some compounds block conduction without depolarizing (DFP, eserine, the tertiary derivative of prostigmine, *d*-tubocurarine, and procaine); others block with depolarization (carbamylcholine, acetylcholine, and dimethylaminoethyl acetate). They suggest that compounds of the first group owe their blocking action to competition with acetylcholine for the same receptor. This suggestion marks a rather radical change in Nachmansohn's views, in that it seems to accept that DFP blocks axonic transmission by another mechanism other than by inhibiting cholinesterase, thereby permitting acetylcholine to accumulate. Later work from his laboratory presenting a detailed analysis of the effect of acetylcholine on electroplax also gives data "at variance with those which one would expect on the basis of the hypothesis proposed by Nachmansohn."[4] Contrary data was later presented, however, using a single electroplax preparation.[51]

Having presented some pros and cons on the role of cholinesterase in axonic transmission, the author would like to be excused from offering a final verdict. Both sides have some convincing arguments, both are suspect of experimental shortcomings. Unfortunately, the acrimonious nature of the disagreement between the two schools of thought has prevented genuine collaboration in an attempt to solve this important problem. All this present account can hope to do is give an idea of what the argument is about and what lines of attack have been employed. This may form a background against which we may watch for further developments to produce a final judgement at whose nature it would be unwise to guess at present.

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* Quoted in reference 47.

Happily the problem will not intrude too much upon our interpretation of events in this book. Only certain anticholinesterases affect axonic transmission, and the over-all picture of poisoning in these compounds appear in no way different from those compounds which certainly do not affect axonic transmission. Therefore we may take it that it is unnecessary to invoke axonic effects in order to account for poisoning by organophosphates.

Let us consider briefly how the insect nervous system compares with the mammalian nervous system, considering the cockroach as the type insect (mainly because a great deal of work has been devoted to it).

(*a*) The central nervous system of the cockroach is less compact than that of the mammal; it is condensed into a series of ganglia. These ganglia are not analogous to the mammalian peripheral ganglia (which are only sites of synapsing of the autonomic system) but to the mammalian brain and spinal cord. Like them, the insect ganglia contain the coordinating systems, and are protected from the influx of many foreign substances. Thus the mammalian central nervous system is protected by the "blood-brain barrier"[22] and the insect ganglia by a connective tissue sheath.[53, 54]

(*b*) The mammalian nerves are of two kinds, "myelinated" and "nonmyelinated." A myelinated nerve is invested by a sheath of a lipid material called myelin, a "nonmyelinated" nerve has only a thin myelin covering. The postganglionic sympathetic fibers are nonmyelinated, all others are myelinated. In the insect, all the nerves are nonmyelinated.

(*c*) The mammalian motor neuron (whose axon carries impulses to the muscle) terminates in a motor end-plate in the voluntary muscle. This end-plate is a relatively bulky expansion of the axon, and contains most of the cholinesterase of the muscle. One "motor unit" of muscle fibers contains one end-plate. In the insect there is no single-axon end-plate. Whole muscles are innervated by only a few axons, which branch freely, then subdivide into "twigs," of which several may go to each muscle fiber. There are several endings on each muscle fiber, some of which are in the form of conical projections: "Doyeres hillocks," also known as end-plates. In the extensor tibialis of the locust these end-plates are distributed 50 to 100 μ apart over the full length of the fiber.[36] These end-plates are not cholinergic.

(*d*) The insect has an autonomic nervous system which controls heart, gut, spiracles, etc. There does not seem to be (as in the mammal) two opposing systems, but only one, which is described as the sympathetic (or visceral) system. Unlike the mammalian system, it has no peripheral synapses and hence no peripheral ganglia.

It is important to note that the mammalian ganglia, which contain peripheral synapses of the sympathetic system and probably are particularly sensitive to organophosphates, have no analogy in the insect. As mentioned before, the insect ganglia are analogous to the mammalian spinal cord and brain.

(*e*) It cannot be stated for certain that insect nerves are cholinergic. Certainly all insects studied contain considerable quantities of acetylcholine, cholinesterase, and choline acetylase, and a good deal of indirect evidence supports the idea that cholinesterase inhibition leads to nerve disruption. The question will be discussed in detail in Chapter 6.

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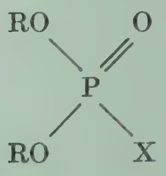
CHAPTER 2

Nonenzymic Reactions*

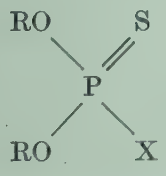
This subject will not be dealt with exhaustively, because the intention is to present only the facts of importance to the biochemical or physiological worker. It is essential that he know how these compounds will behave in common solvents, particularly water; how readily they will break down or isomerize to give compounds having radically different properties from the parent. On the practical side, then, one wants to know what will happen on storage or in a reaction mixture. But as will be seen in Chapter 3, the reactivity of these compounds with cholinesterase is very closely related to their chemical reactivity, as one would indeed expect, since the process of organophosphate inhibition of cholinesterase is a purely chemical one, although very complex. For this reason, a study of some of the mechanisms and equilibria of certain nonenzymic reactions, and of hydrolyses in particular, will throw light on the even thornier problems of enzymic interactions.

Hydrolysis

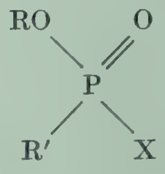
The great majority of toxic organophosphates are esters of phosphoric, phosphorothioic, or phosphonic acids, or of their anhydrides, halides, or amides. All are, therefore, potentially hydrolyzable—the most labile bonds being the anhydride or halide, the next the alkoxy, then the amide. As will be seen, this order of lability depends, however, upon such factors as pH. Some of the newer compounds also have carboxyester or carboxyamidic linkages, and these may be expected to be reasonably labile. Most of the following discussion will be concerned with compounds of the following types:



Phosphate



Phosphorothionate



Phosphonate

where R and R′ are alkyl groups, and X may be a halide, another substituted phosphorus acid linked by an anhydride bond, an alkoxy group or an

* Those unfamiliar with electronic concepts of reaction mechanisms may find Appendix 1 of assistance in this chapter.

aryloxy group, etc. It is in variations of X that the greatest imagination has been lavished, as will be seen by a glance at Appendix 2.

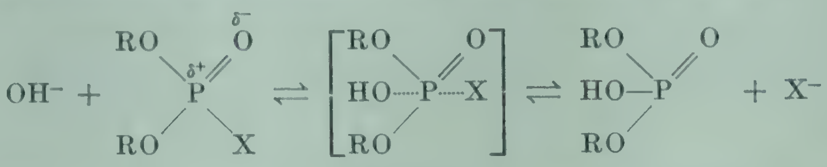
The hydrolysis, both of the RO and the P—X bond under alkaline conditions, has, in most cases examined,[22, 23, 38, 39] been found to be catalyzed most readily by OH^-, and to be of the S_N2 type. That is to say: (*a*) it is a nucleophilic substitution reaction in which the nucleophilic OH^- substitutes for the group X

$$(RO)_2P(O)X + OH^- \rightarrow (RO)_2P(O)OH + X^-$$

(*b*) it proceeds by a bimolecular mechanism, the rate being proportional both to the concentration of $(RO)_2P(O)X$ and of OH^-

$$\text{rate} = k[(RO)_2P(O)X][OH^-]$$

and (*c*) there is no formation of a stable intermediate—the OH^- approaches the molecule and attacks the P because the latter has been made electrophilic by the inductive effect of the =O, and in some of the attacked molecules X^- is released and simultaneously OH combines with the P



On this last point, it should be said that one cannot positively exclude an alternative scheme: that a true intermediate is slowly formed (this being the rate-determining step) and, once formed, breaks down promptly to give products. It would be very difficult to exclude such a mechanism by the current kinetic or isotope exchange techniques.[23]

The factors that influence the hydrolysis rate are: (*a*) variations in the substituents of the phosphorus compound; (*b*) the presence of various catalytic agents; (*c*) variations in the solvent used (hydrolysis is only a special case of solvolysis, which occurs with many other polar solvents besides water); (*d*) variations in temperature and pH.

(1) *Effect of Substituents.* In general, the variations in substituents have the effects predictable on the basis of the mechanism described above: substituents which are electrophilic tend to withdraw electrons and so make the P atom more electrophilic and more liable to OH^- attack, i.e., less stable. Nucleophilic substituents, by contrast, confer greater stability. Before giving examples of these effects, it should be noted that steric effects may in some circumstances obscure the electronic effects: thus, a branched alkyl group may be bulkier than a longer but unbranched group and thereby protects the P atom better from OH^- attack, i.e., stabilizes the molecule. Steric effects should also be expected with any long R chains, and in some

TABLE 2.1. EFFECT OF SUBSTITUENTS UPON HYDROLYZABILITY IN SARIN ANALOGS[a]

$(RO)(R')P(O)F$

R	R′	k_2 (25°C.) 1 mole^{-1} sec.$^{-1}$	E (energy of activation) kcal
1. CH_3	CH_3	106	10.5
2. C_2H_5	CH_3	61	11.2
3. BrC_2H_4	CH_3	162	11.3
4. $C_2H_4\overset{+}{N}(CH_3)_3$	CH_3	935	9.0
5. n-C_3H_7	CH_3	54	10.4
6. $C_3H_6\overset{+}{N}(CH_3)_3$	CH_3	305	11.6
7. *iso*-C_3H_7	CH_3	26	9.1
8. $CH(CH_3)CH_2\overset{+}{N}(CH_3)_3$	CH_3	381	10.1
9. $(CH_3)_2C_4H_8$	CH_3	49	12.0
10. iso-C_3H_7	C_2H_5	9	10.7
11. iso-C_3H_7	iso-C_3H_7	2	9.2

[a] Data of Larsson.[56] Hydroxyl catalysis at pH 8–9.

of the cases were the X group is very large (e.g., Diazinon, Guthion, Co-ral), this effect might outweigh the electronic effects.

The effects of varying R can be shown best in a series of compounds in which X is kept constant. Table 2.1 shows the effect of structure upon hydrolyzability of some analogs of the phosphonate sarin. A large rate constant, k_2, means an unstable molecule. Thus, the following alterations in R involve an increase in the electrophilic nature of the substituents with a production of a less stable molecule: methyl in place of ethyl (1 and 2); bromoethyl in place of ethyl (3 and 2); trimethylammonium in place of H (4 and 2, or 6 and 5, or 8 and 7). And in R′, increasing the size from methyl (7), to ethyl (10), and then isopropyl (11), gives an increasing stabilizing effect to match the increasing nucleophilic nature of R′.

Even more striking was the way in which Larsson[56] was able to compute the hydrolysis constants for some sarin analogs, using the Kirkwood-West-

TABLE 2.2. THEORETICAL AND OBSERVED VALUES FOR HYDROLYSIS RATE CONSTANTS OF PHOSPHONOFLUORIDATES[a]

Compound	Theoretical k_2	Observed k_2
1. 2-bromoethyl methylphosphonofluoridate	2.33	2.21
2. cholinyl methylphosphonofluoridate iodide	3.84	3.29
3. homocholinyl methylphosphonofluoridate iodide	3.18	2.80
4. 1-methylcholinyl methylphosphonofluoridate iodide	3.77	2.90

[a] Theoretical k_2 derived from calculated k_2/k^1_2, where k^1_2 is rate constant for parent compound. From Larsson.[56]

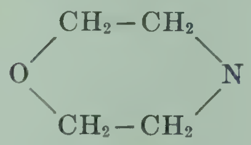
Parent of *1* and *2*: ethyl methylphosphonofluoridate.
Parent of *3*: propyl methylphosphonofluoridate.
Parent of *4*: isopropyl methylphosphonofluoridate.

heimer treatment. In this method, one calculates by classic physics the amount of work done in replacing the group X in, say, $(RO)_2P(O)X$ by OH. The calculation requires a reference compound whose k_2 is accepted, then one may compute the alteration in k_2 produced by altering R. A number of assumptions have to be made about the geometry of the molecule and the dielectric constant of the volume across which the electrostatic effects are transmitted. As such assumptions can only be approximate, precise agreement of prediction and observation is not to be expected. As Table 2.2 shows, Larsson's calculations have given very acceptable estimates of the hydrolysis constants; such work does much to confirm the validity of our ideas upon the factors influencing hydrolyzability.

Table 2.3 shows similar effects in phosphofluoroamidates. Increasing the alkyl size in the monoalkylamide series of R from methyl to ethyl to isopropyl gives progressive stabilization (numbers 2, 3, and 4); similarly in the dialkylamides of R′, diethyl is more stable than dimethyl.[7, 9] However, when R is isopropylamido the P—F bond is more stable than when R is *n*-butylamido, presumably as a result of the steric protection afforded by the branched chain of the iso group.

Fukuto and Metcalf, in an elegant study,[30] have put these electrophilic effects upon a quantitative basis in the case of a series of substituted diethyl phenyl phosphates. Figure 2.1 shows the relationship between hydrolysis constants and Hammet's σ coefficients. These latter are a measure of the electrophilic effects for various substituents: a substituent having a large σ

TABLE 2.3. EFFECTS OF SUBSTITUENTS UPON HYDROLYZABILITY IN PHOSPHORODIAMIDOFLUORIDATES[a]
(RR′POF)

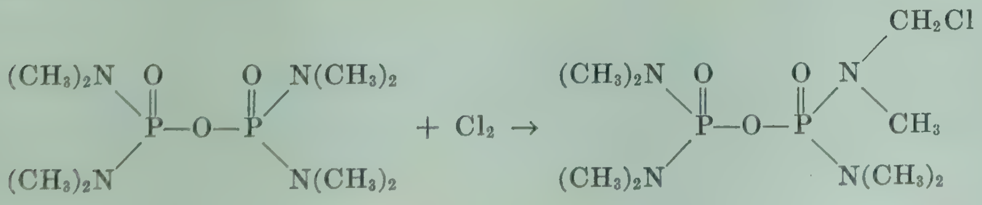
R	R′	k_2 (25°C.) 1 mole^{-1} sec.$^{-1}$	E kcal.
1. n-C_3H_7NH	n-C_3H_7NH	49	11.2
2. CH_3NH	$(CH_3)_2N$	18	11.2
3. C_2H_5NH	$(CH_3)_2N$	12	11.4
4. iso-C_3H_7NH	$(CH_3)_2N$	8.4	11.9
5. n-C_4H_9NH	$(CH_3)_2N$	11	11.4
6. CH_3NH	$(C_2H_5)_2N$	1.7	—
7. 	$(C_2H_5)_2N$	1×10^{-3}	—
8. $(C_2H_5)_2N$	$(CH_3)_2N$	2.9×10^{-4}	16.6
9. $(C_2H_5)_2N$	$(C_2H_5)_2N$	2.5×10^{-5}	17.1

[a] Data of Heath.[38] Hydroxyl catalysis in alkali.

constant is strongly electrophilic. Unfortunately only 7 compounds were examined for hydrolyzability, as having the most readily analyzed hydrolysis products of the 24 compounds synthesized. The correlation of k with σ is quite good, except for the case of the m-dimethylamino compound, which has a negative σ constant.

An important factor in organophosphate chemistry is the change involved in oxidizing P═S to P═O. Table 2.4 shows that the oxidation is always accompanied by decreased stability, as one would expect from the greater electrophilic effect of ═O compared to ═S. The data support the earlier observations of Ketelaar *et al.*[48]

The effect of introducing different numbers of electrophilic groups into one compound was studied in the case of schradan.[78] Up to four chlorine atoms could be introduced, e.g.,



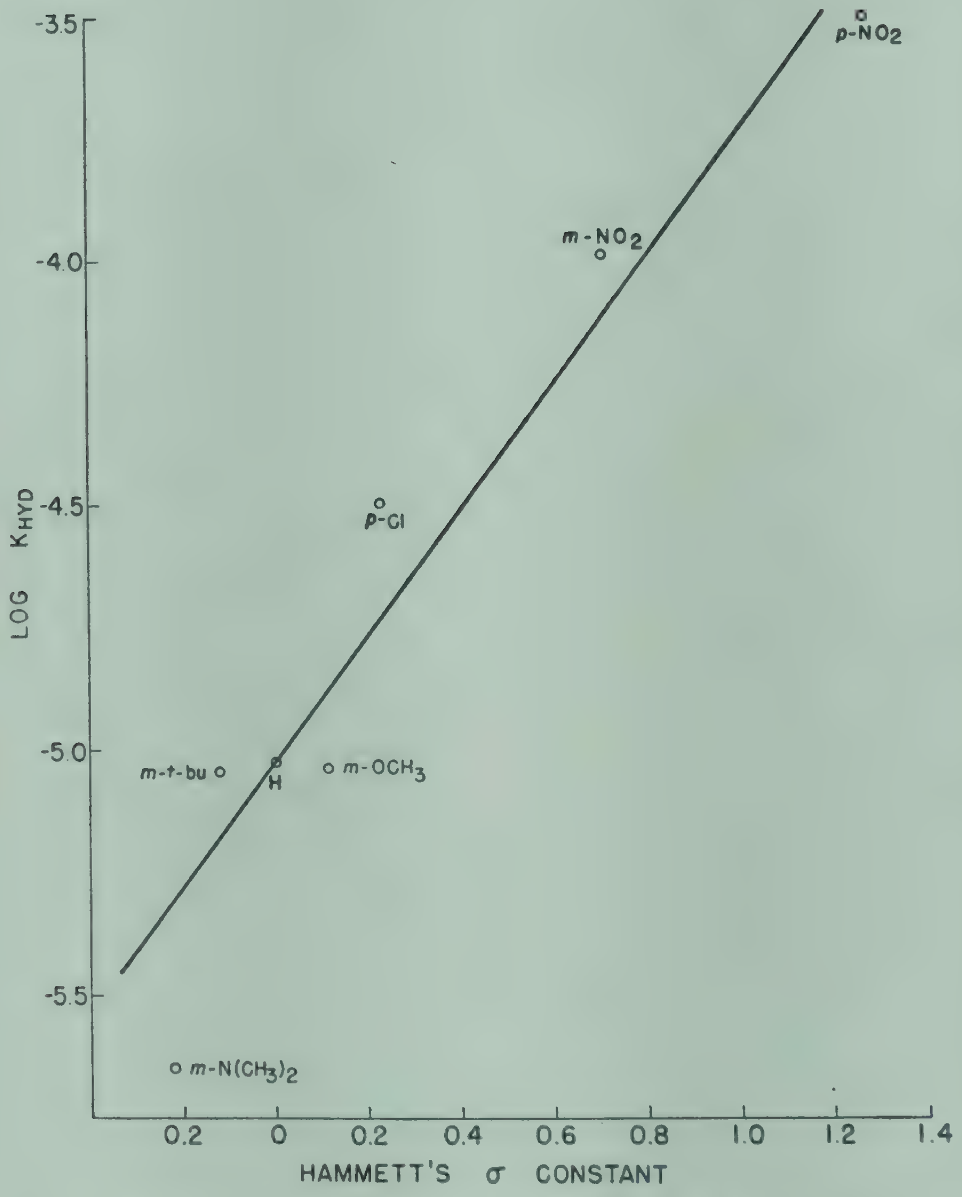
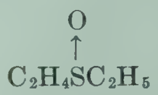


FIG. 2.1. Relationship between hydrolyzability (as log of hydrolysis constant, k) and Hammett σ constant, in substituted diethyl phenyl phosphates. From Fukuto and Metcalf.[30]

It was found that the stability to alkali was greatly reduced by this chlorination: at pH 9 and 15°C., schradan itself hydrolyzes immeasurably slowly, monochlorschradan has a half-life of 118 minutes, dichlorschradan of 15 minutes, and trichlorschradan of 2.6 minutes.

So far, all the evidence described has shown that the stability of $(RO)_2P(S \text{ or } O)X$ at the P—X link is entirely accounted for by the inductive and steric properties of R and X. There are some important exceptions to this rule. Heath[38] points out that where X is a thioester, rates may be

TABLE 2.4. EFFECT UPON HYDROLYZABILITY OF SUBSTITUTING P=O FOR P=S[a]
$(RO)_2P(S\text{ or }O)OR'$

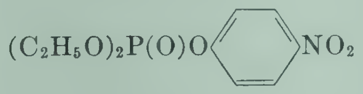
R	R′	k_2 (25° C.)		Mole % hydrolysis pH 7.9, 37°C., 60 minutes	
		=O	=S	=O	=S
p-$C_6H_4NO_2$	CH_3	34	1.4	—	—
C_2H_5	p-$C_6H_4NO_2$	0.52	5.7×10^{-2}	—	—
p-$C_6H_4NO_2$	C_2H_5	4.3	0.4	—	—
C_2H_5	$C_2H_4SC_2H_5$	—	—	39	9.4
C_2H_5	 $\overset{\overset{O}{\uparrow}}{C_2H_4SC_2H_5}$	—	—	13.7	6.2

[a] Data of Heath[38] and Fukuto *et al.*[31]

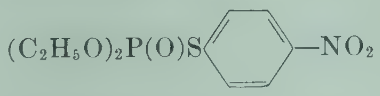
unexpectedly high, and gives examples: (*a*) thiolo-Systox is hydrolyzed more rapidly than paraoxon although p-nitrophenol is a stronger acid than ethylthioethanethiol (which is another way of saying that the p-nitrophenoxy group is more electrophilic than ethylthioethoxy), (*b*) parathion *S*-phenyl isomer is hydrolyzed as fast as TEPP, although diethylphosphoric acid is a stronger acid than p-nitrothiophenol. But Fukuto *et al.*[31] have data on closely related compounds which do not conform with Heath's observations. Thus, $(C_2H_5O)_2P(O)OC_2H_4SC_2H_5$ is hydrolyzed twice as fast as the P(O)S analog; and $(C_2H_5O)_2P(O)OC_2H_4S(O)S_2H_5$ is hydrolyzed 8.5 times

$(C_2H_5O)_2P(O)SCH_2CH_2SC_2H_5$

Thiolo-Systox



Paraoxon



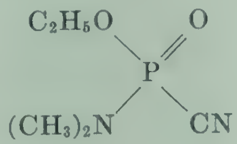
Parathion *S*-phenyl isomer

$(C_2H_5O)_2P(O)OP(O)(OC_2H_5)_2$

TEPP

faster than its P(O)S analog. Obviously more extensive work is needed to clarify this point, and it will probably be best directed to comparing esters with thiolesters for closely related compounds.

Another effect unexpected from simple inductive considerations is the ready hydrolyzability of tabun,[38] $k_2 = 6.3 \times 10^6$.

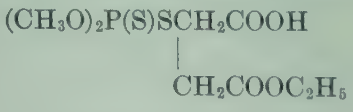


Tabun

Heath points out that this compound is hydrolyzed 10^7 times as fast as paraoxon, although the dimethylamino group is nucleophilic whereas the ethoxy group is electrophilic; and —CN is less electrophilic than *p*-nitrophenol, since it forms a weaker acid.

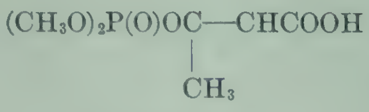
There are other exceptions. Some may be explained by the greater inductometric polarizability of S as compared to O, or of C═N as compared to alkoxy: this is an effect which comes into play only during the transition state, when the OH^- is attacking the P atom. But as we have at present no reliable information as to which way hydrolyzability will be changed on altering P—O—C to P—S—C (for instance), it would be idle to speculate at greater length.

It should be quite safe to predict that when the newer compounds containing carboxyester and carboxyamide linkages are split (whether enzymically or chemically) the resulting carboxylate ion should strongly depress subsequent hydrolyses in the molecules, as a result of the strong nucleophilic effect of an anion. Thus, one would expect the carboxyacid derivatives of malathion or dimethoate to be much more stable than their parent compounds; and the carboxyacid derivative of Phosdrin has indeed been shown to be more stable than Phosdrin.[81]



$$(CH_3O)_2P(S)S\underset{\substack{|\\ CH_2COOC_2H_5}}{CH_2}COOH$$

Malathion monoacid



$$(CH_3O)_2P(O)O\underset{\substack{|\\ CH_3}}{C}—CHCOOH$$

Phosdrin acid

$(CH_3O)_2P(S)SCH_2COOH$

Dimethoate acid

Heath[39] has studied the hydrolysis of a number of organophosphates at low OH^- concentrations. We shall consider, first, those which do not contain nitrogen.

The evidence is that hydrolysis of the P—X bond is accomplished under neutral or acid conditions by water acting as an anionoid reagent, i.e., act-

TABLE 2.5. AQUEOUS HYDROLYSIS OF PYROPHOSPHATES[a]
$(RO)_2P(O)OP(O)(OR')_2$

R	R′	First-order rate constant k_1, 25°C. water
CH_3	CH_3	25×10^{-3}
CH_3	C_2H_5	7.0×10^{-3}
CH_3	$n\text{-}C_3H_7$	5.6×10^{-3}
CH_3	$\text{iso-}C_3H_7$	1.1×10^{-3}
C_2H_5	C_2H_5	1.6×10^{-3}
C_2H_5	$n\text{-}C_3H_7$	1.0×10^{-3}
C_2H_5	$\text{iso-}C_3H_7$	$0.28 \times 10\ ^{3}$
C_2H_5	$n\text{-}C_4H_9$	0.95×10^{-3}
$n\text{-}C_3H_7$	$n\text{-}C_3H_7$	6.5×10^{-4}
$\text{iso-}C_3H_7$	$n\text{-}C_3H_7$	2.0×10^{-4}
$\text{iso-}C_3H_7$	$\text{iso-}C_3H_7$	0.9×10^{-4}
$\text{iso-}C_3H_7$	$n\text{-}C_4H_9$	2.0×10^{-4}

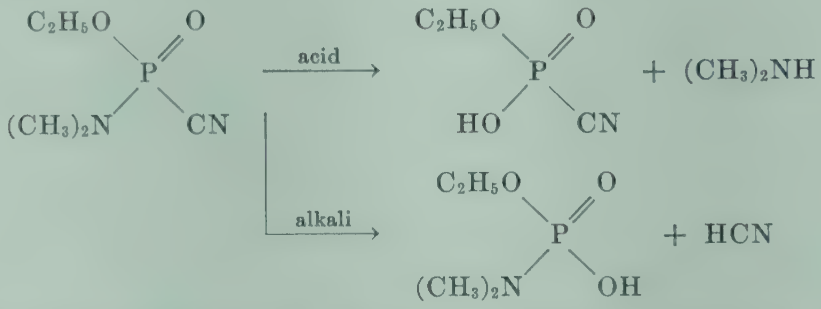
[a] Data of Heath.[39]

ing as a sort of modified hydroxyl ion. The mechanism is S_N2 as in alkaline conditions, and one expects the effect of substituents to be the same as described above. Table 2.5 shows that the expectation is fulfilled: for pyrophosphates, if any R is held constant and the size of R′ increased, the compounds are progressively more stable; except that (as in alkaline conditions) the isopropyl group gives a more stable compound than *n*-butyl, probably because of a steric effect. However, for the case of DFP there is fairly strong evidence[50, 89] that at neutral and lower pH, acid catalysis is the predominant mechanism.

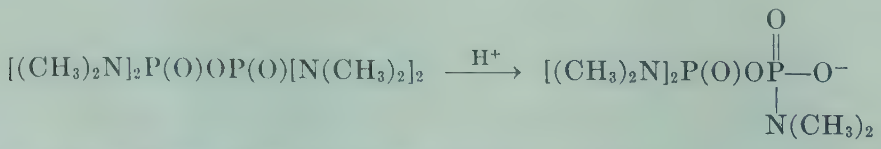
In the case of pyrophosphates, i.e., $(RO)_2P(O)OP(O)(OR)_2$, Heath argued that "both phosphate groups in each molecule are open to attack," and therefore the over-all hydrolysis constant would be the sum of those for each half. His constants calculated on this basis agreed with those observed only in 2 out of 8 cases, a discrepancy which Heath attributed to steric effects, which would, thus, be more severe than in single-phosphorus compounds. Brock,[9] however, showed later that the observed constants agreed excellently in every case if one summed the effects of the individual substituents upon a hypothetical single hydrolysis. He therefore suggested that the pyrophosphates must be attacked singly and probably centrally, on the oxygen of the P—O—P, rather than being open to attack at both ends on the phosphoruses. However E. Y. Spencer (private communication) suggests that Brock's calculations fit well with the hypothesis that one or other phosphoruses is in fact attacked; the successful attack upon one would greatly decrease the susceptibility of the molecule to further hydrolysis and it is, therefore, incorrect to treat a pyrophosphate as if it had two independant ends, each susceptible to hydrolysis. Spencer's proposal has the virtue of not requiring separate theories for the hydrolyses of phosphates and pyrophosphates.

The hydrolysis of the N—P bond in phosphoramidates proceeds by a different mechanism from that of the P—X bond just described. Heath and Casapieri[40] showed that the P—N bond is very stable in alkali, but unstable in acid. Therefore catalysis is by H^+ rather than OH^- in this case. Under neutral conditions measurable P—N hydrolysis is only found in phosphoramides which are not fully substituted, and so contain the N—P—OH group.

Similarly, Larsson[54] showed that the hydrolysis of tabun in an unbuffered system (which presumably would be about pH 5 or 6 and become progressively more acid as the reaction proceeded) the dimethylamino group was split off, as judged by infrared and free dimethylamine analyses. Under alkaline conditions by contrast, Holmstedt[44] showed that the cyanide group was split out.

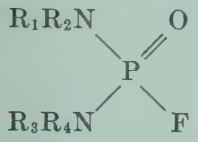


That phosphoramides should be attacked by the electrophilic H^+ rather than by the nucleophilic OH^- is to be expected, since NRR′ has a very weak electrophilic effect compared to OR, particularly when R and R′ are alkyl groups rather than H. The phosphorus in the phosphoramides is, therefore, much less electropositive than in the phosphate esters, and so less susceptible to attack by the "positive-seeking" nucleophile. It is also possible to conceive that the free electrons on the nitrogens may repel somewhat the negative hydroxyl ion, thus limiting its access to the phosphorus. Once the P—N link is cleaved in (say) schradan, the resultant negative charge (at pH values near or above the pK value), makes all the other bonds susceptible to electrophilic attack, and degradation to orthophosphoric acid occurs rapidly.[40]

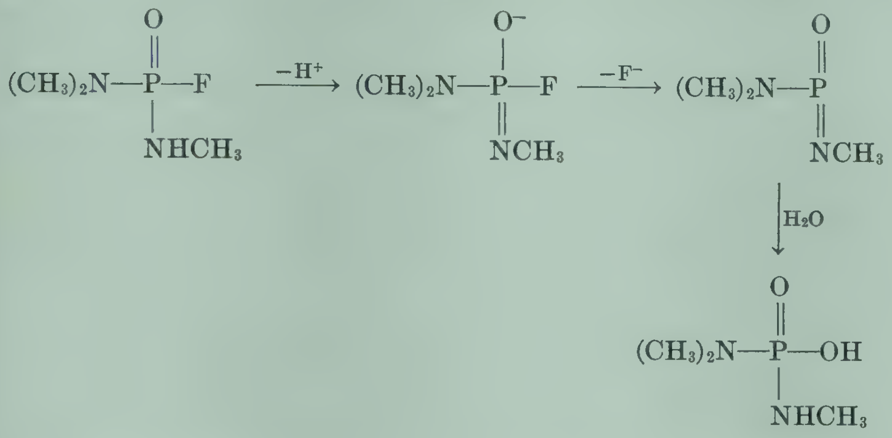


$$[(CH_3)_2N]_2P(O)OP(O)[N(CH_3)_2]_2 \xrightarrow{H^+} [(CH_3)_2N]_2P(O)O\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle N(CH_3)_2}{|}}{P}}—O^-$$

There is evidence that in those phosphoramidates containing one or more hydrogens attached to a nitrogen, a special form of alkaline hydrolysis occurs. Heath[38] pointed out that in compounds of the type

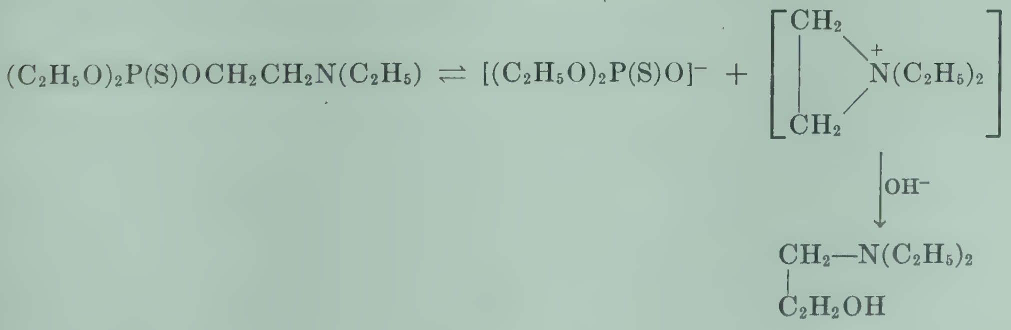


when one of the R groups was hydrogen, a great enhancement of alkaline hydrolyzability was found. He attributed it to a steric effect. Westheimer[91] suggested that a special dehydrogenation could occur, e.g.,



This proposal is supported by: (*a*) the fact that in such cases the base lutidine had a particularly marked catalytic effect,[19] presumably by binding the removed proton; (*b*) the marked sensitivity to alkaline hydrolysis of related —NH-containing phosphoramidates.[75]

(2) *Site of Cleavage.* A question that arises in the hydrolysis of triesters of phosphoric acid is: does the cleavage occur at the P—O or the O—R bond? For a number of cases it has been shown that in alkaline hydrolysis it is the P—O bond that is broken.[16, 86] Under neutral conditions O—R cleavage usually predominates.[86] However, when the P—S—C of malathion is hydrolyzed in alkali, it is the S—C that is cleaved, since *O,O*-dimethyl phosphorodithioic acid is produced as judged by the color given with Cu^{++} by the product[66] and by polarographic analysis.[46] Tetram is also cleaved by alkali at the O—C bond, probably because the compound is usually in equilibrium with an immonium ion intermediate:[28]

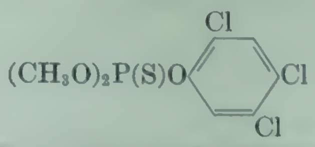


Substantiating evidence that it is the presence of the immonium intermediate which causes the O—C cleavage is the fact that the thiolo isomer of Tetram (containing the group P(O)SC), which gives rise far less easily to the immonium ion,[32] is split by alkali at its P—S rather than its S—C bond.[28]

In the account so far, mention has been made only of the hydrolysis of P—O—X in compounds of the type $(RO)_2P(O)OX$ and $(RO)RP(O)OX$. It was mentioned, however, that compounds containing the R′N—P group were cleaved at the N—P in acid solution. Plapp and Casida[72] have recently shown that in the alkaline hydrolysis of ronnel in 95% ethanol, cleavage occurs at the CH_3—O—P or the P—O— phenyl linkages, depending upon conditions, as shown in Table 2.6. These authors[73] also studied the phenomenon in a number of other common aromatic phosphates under the same conditions, and found substantial alkoxy-phosphorus cleavage in all cases; the methoxy compounds were particularly readily hydrolyzed at the P—O—CH_3 (Table 2.7). They used both organophosphate and KOH at 25 mM, in 95% ethanol (this KOH concentration in water would give a pH of about 12.4). However, when these compounds were hydrolyzed "in 50% ethanol with strong KOH," there was never more than 10% of the hydrolysis products which were cleaved at the P—O—alkyl. Unfortunately, one cannot say if the change in ethanol or in alkali level caused this different behavior; but it is clear that the problem is a complex one, and that the factors determining the place of cleavage are not well understood. It is probably desirable to use, where possible, a very large ratio of alkali to organophosphate, and study various concentrations of alkali; under equimolar conditions, the alkali concentration changes as the reaction proceeds.

(3) *Secondary Hydrolysis.* So far, we have discussed only hydrolysis of fully

TABLE 2.6. VARIATIONS IN PLACE OF CLEAVAGE OF RONNEL IN VARIOUS ALKALINE CONDITIONS[a]



Condition	Ronnel		Oxygen analog of ronnel	
	% Cleaved at P—O—CH_3	% Cleaved at P—O—ph	% Cleaved at P—O—CH_3	% Cleaved at P—O—ph
1 Mole KOH	78	22	49	51
2 Moles KOH	64	36	25	75
4 Moles KOH	4	96	2	98

[a] Data of Plapp and Casida.[72]
Compounds in 95% ethanol, 28° C. for 16 hours.
Figures are % of total hydrolysis products.

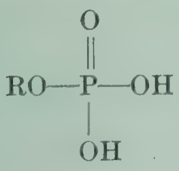
TABLE 2.7. FRACTION OF TOTAL HYDROLYSIS PRODUCTS CLEAVED AT P—O—ALKYL IN VARIOUS SUBSTITUTED PHENYL PHOSPHATES[a]
(RO) P(O or S) OphX

Compound	R	=O or =S	X	% of hydrolysis in 20 hours (total products)	% of hydrolysis products cleaved at P—O—R
Parathion	C_2H_5	=S	4-NO_2	9	11
Paraoxon	C_2H_5	=O	4-NO_2	41	12
Methyl parathion	CH_3	=S	4-NO_2	33	46
Am. Cy. 4124	CH_3	=S	2-Cl,4-NO_2	35	46
Chlorthion	CH_3	=S	3-Cl,4-NO_2	35	35
Ronnel	CH_3	=S	2,4,5 Cl_3	46	64
—	CH_3	=O	2,4,5 Cl_3	69	49
—	C_2H_5	=O	2,4,5 Cl_3	38	29
Diazinon	C_2H_5	=S	Not phX but 2-isopropyl-4-methyl-6-pyrimidinyl	13	9

[a] Data of Plapp and Casida.[73]

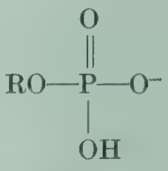
esterified phosphorus acids: the main reason for this narrow interest is that hydrolysis of the first ester constitutes detoxification (for very good reasons that will appear in the next chapter) so that further hydrolysis is of little importance as far as toxicity is concerned. However, the hydrolysis of partially esterified phosphates has been extensively studied because of its complexity and physicochemical interest. Since studies of the routes of biological degradation of the toxic organophosphates involve the isolation of the partial esters, it is important to know a little about their expected stabilities. Fortunately, as far as phosphates go, the primary phosphoric hydrolysis products of a very great proportion of the interesting compounds comprise only dimethyl, diethyl, and diisopropyl phosphate: $(RO)_2POOH$. With phosphorothioates and dithioates we must add the corresponding compounds $(RO)_2PSSH$ and $(RO)_2PSOH$.

First, a brief mention is deserved for the curious pH dependence of hydrolysis of many monoesters $(RO)P(O)(OH)_2$. An example is given in Fig. 2.2, from which it is apparent that there is a maximum of instability at pH 4. This behavior is frequent with such monoesters, not in the diesters. Now a monoester can exist in various forms, depending upon pH:



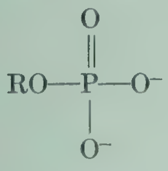
$$RO-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}-OH$$

(I)



$$RO-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}-O^-$$

(II)



$$RO-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}-O^-$$

(III)

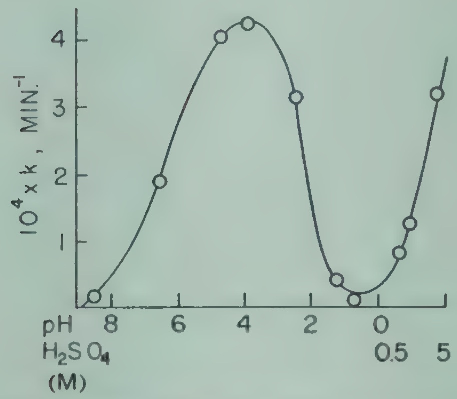
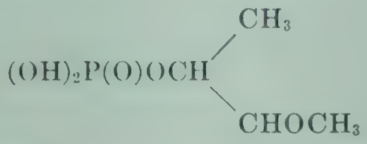


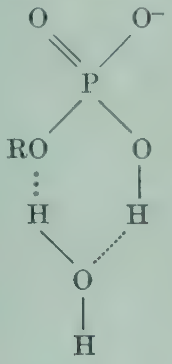
FIG. 2.2. Relationship between pH and hydrolysis constant for 1-methoxypropyl-2-phosphate



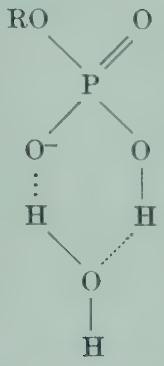
$$(OH)_2P(O)OCH\begin{matrix} CH_3 \\ CHOCH_3 \end{matrix}$$

From Butcher and Westheimer.[10]

Butcher and Westheimer[10] pointed out that the maximum at pH 4 must mean that (II) is a particularly hydrolyzable form, and suggested that this is due to its ability to complex with water, to form:



A better formulation by Vernon[86] was:



This formulation makes use of the crucial single anion, and shows that only type (II) could possibly form such a complex.

TABLE 2.8. EFFECT OF SOME AMINO ACIDS AND RELATED CHEMICALS AT 0.1 M AND pH 9.0 ON THE HYDROLYSIS OF ALPHA-PHOSDRIN

% Hydrolysis	Compounds
27%–34%—Not significantly higher than borate-barbital buffer alone[a]	D-Alanine, L-alanine, DL-homoserine, glutamic acid, tyrosine, imidazole, phenylglycine, L-phenylalanine, β-phenylalanine, tryptophan, DL-citrulline, creatinine, betaine hydrochloride, creatine, hippuric acid, *p*-aminobenzoic acid, 2,5-dinitrophenol, DL-aminobutyric acid, β-amino isobutyric acid, tris-(hydroxymethylaminomethane), 4,5,6 hydroxy-2,7 napthalene sulfonic acid, cobalt chloride, cadmium sulfate, sodium borate, chymotrypsin[b]
35%–55%	L-Isoleucine, D-leucine, DL-norleucine, L-leucine, L-valine, β-alanine, L-threonine, D-serine, L-serine, aspartic acid, dimethylglyoxime, *p*-hydroxybenzoic acid, *p*-aminoethanoic acid
56%–80%	Glycine, DL-allothreonine, L-histidine, DL-histidine, ammonium molybdate, dimethylamine hydrochloride
81%–95%	DL-Proline, L-proline, hydroxylamine hydrochloride, catechol
96%–99%	3,4-Dihydroxyphenylalanine, L-hydroxyproline, pyridine-2-aldoxime methiodide, pyridine-3-aldoxime methiodide, pyridine-4-aldoxime methiodide, pyridine-2-aldoxime dodeciodide

[a] Boric acid and sodium barbital each at 0.05 M and pH 9.0. All other compounds were added to this buffer at 0.1 M and the buffer readjusted to pH 9.0 before adding the alpha-Phosdrin at 500 p.p.m. After 24 hours incubation at 28° C., the pH was rechecked and each mixture was extracted with an equal volume of chloroform and the total phosphorus content of the aqueous phase was considered to be hydrolysis products.

[b] Crystalline chymotrypsin at 1 and 10 mg./ml. at pH 6.5 and 8.5, in the presence of 0.1 M glycine, gave no more Phosdrin hydrolysis than did glycine alone.

Data of Gatterdam *et al.*[33]

An idea of the stability of monomethyl and dimethyl phosphates is given by their half-lives: at pH 3.3 and 100°C., these are 27 hours and 2300 hours, respectively. The greater stability of the dimethyl phosphate is presumably due to its inability to form the type of complex with water, as shown above. The hydrolysis of dimethyl phosphate is acid catalyzed, but even in 5 M perchloric acid at 100°C. its half-life is 320 minutes.[86]

(4) *Catalysis.* The hydrolysis of organophosphates is catalyzed by many compounds, particularly nitrogenous ones. Table 2.8 gives some effects of

TABLE 2.9. CATALYSIS OF DFP HYDROLYSIS BY HYDROXAMIC ACIDS[a]

Catalyst[b]	Half-life of DFP (minutes)
—	Several days
Benzohydroxamic acid	22
Nicotinhydroxamic acid	20
Methiodide of nicotinhydroxamic acid	68
Picolinhydroxamic acid	65
Isonicotinhydroxamic acid	28
Methiodide of isonicotinhydroxamic acid	73

[a] Data of Hackley *et al.*[35]

[b] Catalysts at 8.5×10^{-3} *M*, 30°C., pH 7.6.

substances at high concentrations upon the hydrolysis of *cis*-Phosdrin. The solvolysis of, e.g., tetrabenzyl phosphate by propanol is strongly catalyzed by various bases, particularly collidine and lutidine, and in fact, the rate constant is directly proportional to the base concentration.[90] A catalytic effect of histidine, pyridine, and derivatives upon DFP hydrolysis has been observed,[87] but high concentrations (e.g., 10^{-2} *M*) were used.

More striking effects have been obtained with hydroxamic acids. Hackley *et al.*[35] studied more than 30 of these acids, all of which reduced the half-life of sarin from the uncatalyzed value of 300 minutes down to from 1 to 7 minutes. Table 2.9 shows how these acids also drastically reduced the half-life of DFP. The effectiveness of these catalysts is reduced by quaternerization (Table 2.9) and increases with pH, suggesting that the ionic is the active form. It was shown that the mechanism of the catalysis is by reaction of the phosphate and the hydroxamate, forming an unstable compound which rapidly breaks down to give phenyl isocyanate and the hydrolysis products of the phosphate, e.g.,

$$\text{PhCONHOH} + \text{FP(O)(OR)}_2 \xrightarrow{-\text{HF}} \text{PhCONHO—P(O)(OR)}_2 \rightarrow$$

$$\text{PhNCO} + \text{HO—P(O)(OR)}_2$$

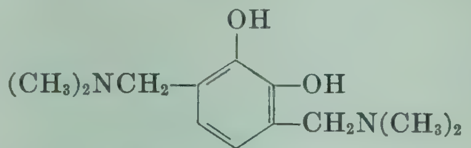
Hackley *et al.*[35] also noted that a number of hydroxyamidines

$$\text{RC(=NH)NHOH}$$

the imino analogs of the hydroxamates, reacted with DFP and sarin; but in this case a stable product was formed (see also p. 105).

The kinetics of oxime and hydroxamate catalysis have been studied by Green *et al.*[33a, b]. The rate of reaction between catalyst and phosphate determines the catalyzed hydrolysis rate, and is particularly influenced by the basicity of the catalyst; for any given phosphate the reactivity with a series of hydroxamates is directly proportional to their pK_a's.

T. Higuchi (private communication) has been able to show that two factors are involved in the catalytic hydrolysis of sarin by salts of certain compounds derived from catechol, e.g.,



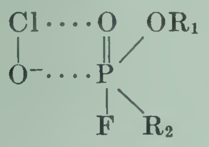
3,6-bis-(Dimethylaminomethyl)catechol

He calculated for each catalyst an equilibrium constant K which measured the tendency for complex formation with sarin, and a rate constant k_2 which measured the reactivity with the sarin. This k_2 was controlled by the basicity of the group which actually attacks the phosphorus.

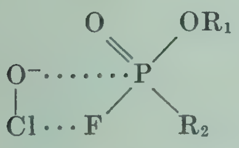
Another catalytic agent that has been studied is chlorine, with respect to its effect on sarin hydrolysis.[25] From the pH dependence of the catalysis it was concluded that the hypochlorite ion, OCl^-, was responsible. The catalytic effect varied directly with the Cl_2 concentration:

$$\text{rate} = k[\text{sarin}][Cl_2]$$

and Cl_2 was not used up by the reaction. Presumably the initial attack upon the P was by the OCl^- rather than the usual OH^-; and since the effectiveness of OCl^- over OH^- was greater than could be accounted for simply by the increased nucleophilic powers bestowed by the Cl upon the O, it was suggested that a bifunctional attack was made:



or possibly



Other ions have been found to catalyze organophosphate hydrolyses. Heath and Casapieri[40] found that although dimefox was unaffected by water at 100°C. in 3 days, in 0.1 N phosphate buffers at 100°C., about 5% hydrolysis occurred in 3 hours. Similarly, mipafox hydrolysis was catalyzed by phosphate-citrate buffer pH 5.7, to an extent depending upon the molarity of the buffer.[37]

Ketelaar *et al.*[47] observed startling catalytic effects of copper upon the hydrolysis of parathion and EPN, and a smaller effect upon paraoxon. With Cu^{++} at $3 \times 10^{-7}\ M$ and the ester at $10^{-4}\ M$, the metal increased the hydrolysis rate 20-fold for parathion and 48-fold for EPN. Other metals

Table 2.10. Acceleration of DFP Hydrolysis by Various Copper Chelates[a]

Complexing agent	Half-life (minutes)	Complexing agent	Half-life (minutes)
No catalyst	>2500	Threonine	18
β-Alanine	29	Ethylenediamine	16
Glycine	27	Imidazole	14
Aspartic acid	26	*o*-Phenanthroline	14
Glutamic acid	25	4,4′-Dimethyl-2,2′-bipyridyl	9
Arginine	23	L-Histidine	8
Lysine	23	α,α′-Dipyridyl	4.5

[a] Conditions: Bicarbonate buffer pH 7.6, 38°C. DFP 4.5×10^{-3} *M*.
Complexing agent and copper 22.8×10^{-3} *M*.

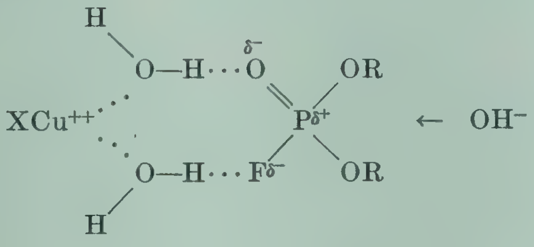
Data of Wagner-Jauregg *et al.*[88]

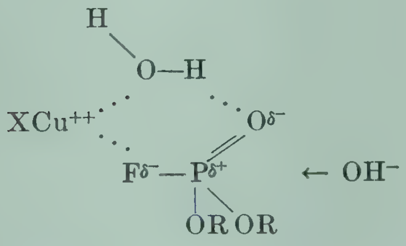
(magnesium, calcium, barium, aluminum, cobalt, cadmium, and silver) were ineffective; but reaction was more rapid in a glass than in a polyethylene vessel. These observations were made at pH 7.8–8.6; under alkaline conditions, copper had no catalytic effect. Ketelaar proposes a complex formation between Cu^{++} and the slightly electronegative sulfur atom, which would lead to an enhanced polarity of the P═S; such a complex, says Ketelaar, should "show more 'opposing resonance' and a reduced stability." Alternatively one could explain it on the basis of an enhanced electrophilic effect of $=S:Cu^{++}$ over ═S, thus, making the P more electropositive and susceptible to anionic attack. The catalytic effect has been confirmed by Sandi.[76] It was shown by Wagner-Jauregg *et al.*[88] that molybdate and cupric ions effectively catalyzed DFP hydrolysis; cobaltous and nickel ions were slightly effective; lanthanum, ferrous, palladous, and chromic ions were without effect.

Copper ion, when chelated with a number of compounds, is extremely effective as a catalyst (Table 2.10). The effect is much in excess of that found with either agent separately. Chelates of nickel and cobalt are much less effective; iron and manganous chelates are ineffective. It seems that copper in combination with agents which tend to saturate the copper's binding capacity makes a poor catalyst: thus, copper-Versene (ethylenediamine tetraacetic acid) is poor, and $Cu^{++}X$ chelate forms are more effective than the $Cu^{++}X_2$ (where X is the chelating agent). Wagner-Jauregg *et al.*[88] suggest that in the $Cu^{++}X$ form, when X is a tetra-coordinating chelator (e.g., dipyridyl), one has at low pH a dihydrate of the unoccupied coordination pair, $Cu^{++}X\ (H_2O)_2$, and that the water molecules may be replaced by hydroxyl at higher pH; thus,

$$\underset{\text{(I)}}{Cu^{++}X(H_2O)_2} \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} \underset{\text{(II)}}{Cu^+X(OH)(H_2O)} \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} \underset{\text{(III)}}{CuX(OH)_2}$$

Which form is the effective catalyst? The plot of pH dependancy (Fig. 2.3) suggests that (III) is the most active species; but (II) dimerizes strongly to an inactive form, so its true effect is not shown by the figure. The authors suggest that the mechanism of action of these copper-chelate catalysts might be through the formation of such complexes with the organophosphate as shown below.





The strongly positive copper, in such a complex, should enhance the elec-

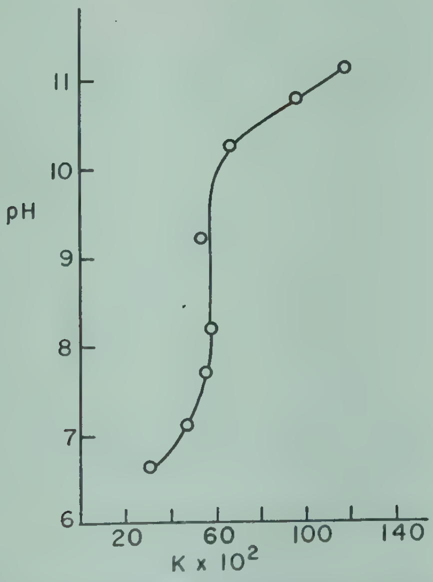
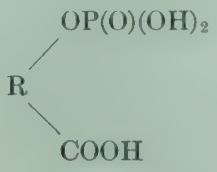
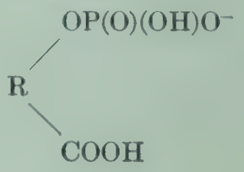


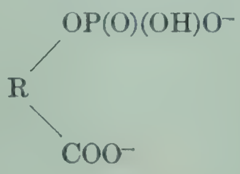
FIG. 2.3. Relationship between pH and hydrolysis constant for DFP in the presence of $CuSO_4$ and dipyridyl (1:1) at 30°C. From Wagner-Jauregg *et al.*[88]

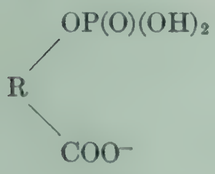
trophilic effect of the F and O, and thus make the phosphorus more positive and more susceptible to attack by the nucleophilic hydroxyl ion.

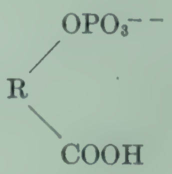
Chanley *et al.* have reported an interesting example of what might be called intramolecular catalysis for the monosubstituted phosphate, salicyl phosphate.[16] Its implications could be important for other phosphates. These authors noted the odd phenomenon that this compound has a peak of hydrolyzability at pH 5.3. Now the possible ionic forms at various pH values are (where R is a phenyl group):

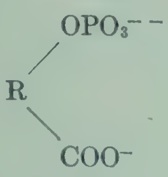

(I)


(II)

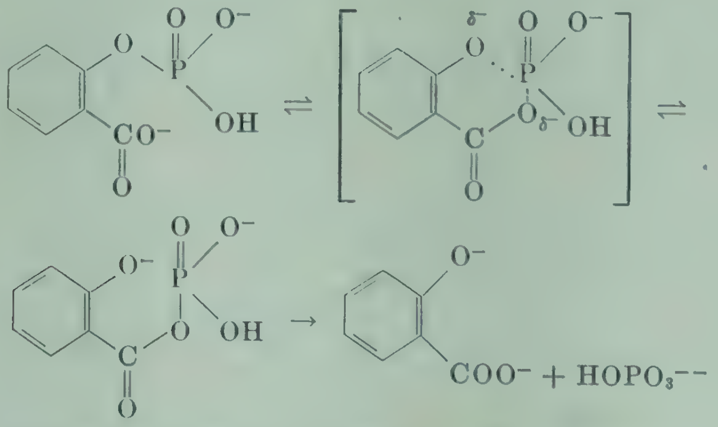

(III)


(IV)

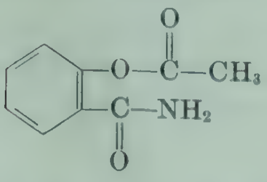

(V)

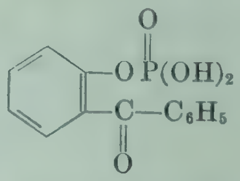

(VI)

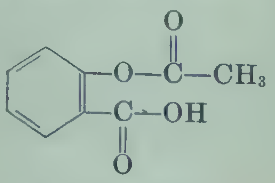
The stability of the compound at extreme pH values implies that (I) and (VI) are stable. A variety of indirect pieces of evidence suggested that (III) and (IV) were the unstable forms. They suggest the following reaction mechanism:



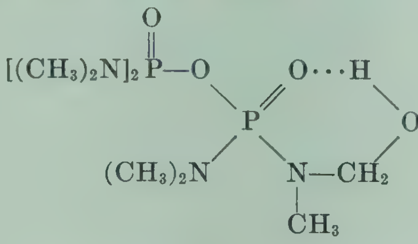
The authors consider that this formation of a cyclic intermediate may also occur in *O*-acetylsalicylamide, salol phosphate, and acetylsalicylate.


O-Acetylsalicylamide

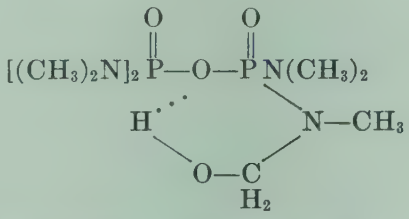

Salol phosphate


Acetylsalicylate

Spencer[77] has suggested that the oxidized form of schradan, whose structure is now considered to be the hydroxymethyl derivative,[41, 80] owes the fact that it is enormously better as an anticholinesterase and more unstable to alkaline hydrolysis than its parent, to a cyclization:

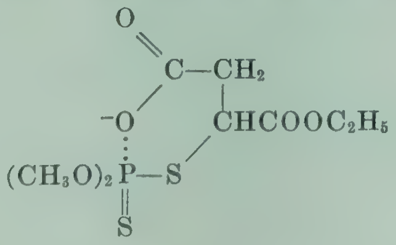


An alternative way to account for the properties of the hydroxymethyl schradan would be by the electrophilic effect of the OH as compared to the H that it replaced, but the observed effect is perhaps too strong to be attributed to the moderately electrophilic effect of an OH, particularly when transmitted through two other atoms (N and C). Instead, using Spencer's formulation, one would argue that the hydrogen bonding would enhance the electrophilic nature of the P by the tendency of the hydrogen to draw an electron away from its neighboring =O. An alternative formulation was proposed by Westheimer[91]:

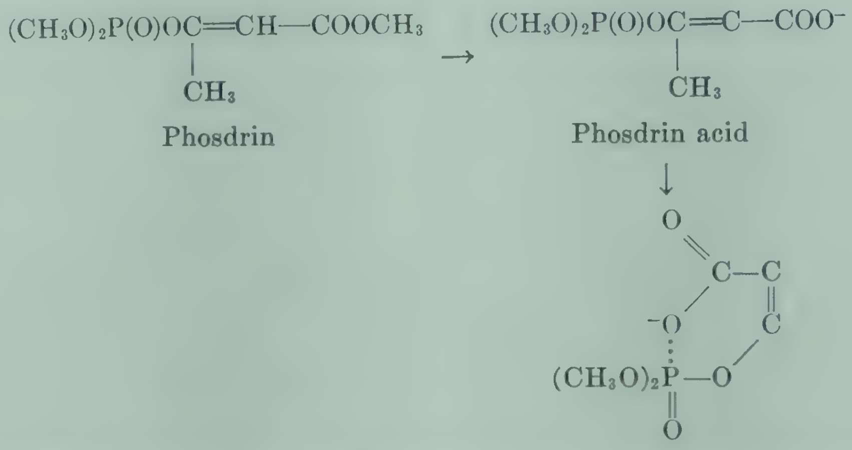


However, this anhydride oxygen is much less likely to form hydrogen bonds than is the more electron-rich phosphoryl oxygen.

Spencer's proposed structure differs from the case of salicyl phosphate in that (*a*) it has hydrogen bonding rather than O^-....P bonding, and (*b*) there are no ionic species present. There are cases in which a bonding quite similar to salicyl phosphate might occur, i.e., in those newer organophosphate anticholinesterases which contain carboxyester or carboxyamides. One must consider the possibility of cyclization, either in the parent compound or in carboxylate-ion-containing degradation products, e.g., one of the two possible malathion acids could be written thus:



Such a formulation was proposed by Spencer *et al.*[81] in a search for possible derivatives of Phosdrin which might be more reactive and less stable than the parent compound. They examined the properties of the hydrolysis product, Phosdrin acid:

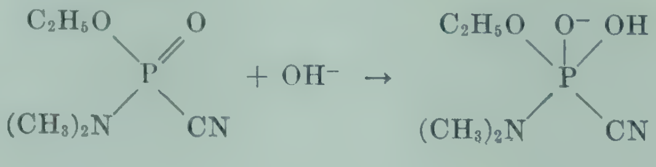


In fact, the Phosdrin acid was much more stable and unreactive than Phosdrin: cyclization was either not present or its effects were swamped by the transmitted or direct nucleophilic influence of the carboxylate ion.

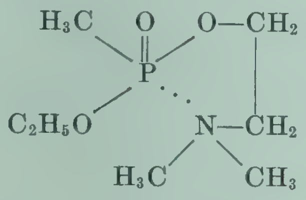
Nevertheless, such cyclizations could well modify the expected properties of such compounds. It remains to be seen, of course, if this can occur with fully substituted phosphates; as pointed out above, cyclization in salicyl phosphate was not very large in the un-ionized form. If one considers only inductive effects, these lie in the order $RO > HO \gg O^-$, so that on this basis the substituted phosphates would be even less inclined to cyclize than the un-ionized unsubstituted form. However, the inductometric polarizabilities lie in the order $O^- > RO > HO$, so that it is possible that in the transition state during the hydrolysis, the substituted phosphate might have an intermediate reactivity between the ionized and un-ionized forms of the unsubstituted phosphate.

Furthermore, recent studies (as indicated above and in Chapter 6) have suggested that one may obtain in certain cases degradation products, both alkaline and enzymic, of the form $(RO)(HO)P(O)OX$, and in some of these cyclizations might occur, if a nucleophilic substituent—an anion or possibly a tertiary nitrogen—were appropriately located.

The possibilities of cyclization have been discussed by Larsson. In an early paper[55] he suggested that in the hydrolysis of tabun, an intermediate might be transiently formed:



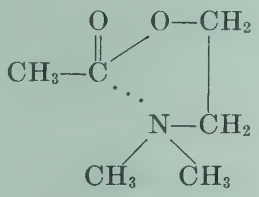
However, in a later paper[56] he argued that if such a complex were possible, then nucleophilic groups other than OH^- should also form one, and, thus, that intramolecular bonding should occur with an appropriately placed nucleophilic group, e.g.,



(I)

Now such a bonding would weaken the basicity of the nitrogen. But, in fact, Tammelin[82] had shown that the basicity of (I) was similar to that of the corresponding acetyl ester, β-dimethylaminoethanol acetate (II). For this reason Larsson rejects the possibility of cyclization.

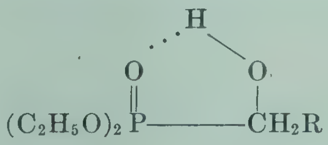
However, in the carbon analog (II) it seems possible that considerable internal bonding could occur:



(II)

In fact, the pK_a of (II) is 8.35, as compared with 9.72 for trimethylamine,[53] and such a difference seems rather too much to attribute to the electrophilic inductive effect of the $CH_3C(O)OCH_2$-substitution. If so, the base weakening effect in (II) may be caused by cyclization, and we have therefore no grounds to reject the possibility of cyclization in the organophosphate.

Another case of internal bonding in phosphorus compounds has been reliably reported. Miller *et al.*[63] have shown on infrared evidence that with aromatic α-hydroxy phosphonates, hydrogen bonding occurs as follows:



where R can be aromatic or aliphatic. Other evidence, with substituted phosphine oxides, suggested that the degree of such bonding would decrease with more electrophilic substitutents than ethoxy. Intermolecular bonding can occur also.

It seems very probable that a similar bonding should occur with Dipterex

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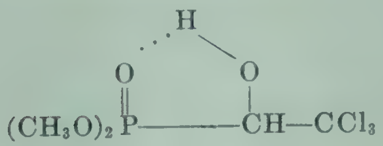
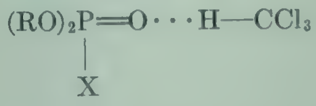


unless the methoxy groups are too electrophilic.

Some assistance in predicting the probability of hydrogen bonding has been provided by Kosolapoff and McCullough,[51] who measured intermolecular bonding between chloroform and organophosphates, using heat of mixing as a criterion (hydrogen bonding should contribute extra heat) e.g.,

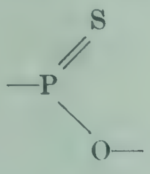


$$\underset{\displaystyle X}{\overset{}{(RO)_2P}}{=}O \cdots H{-}CCl_3$$

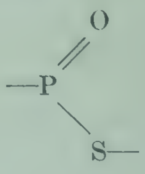
Observation followed prediction: bonding was improved by substituents on P that made it more negative, and was reduced by electrophilic substituents. Thus RO-compounds bonded less than R-compounds but better than Cl-compounds. Comparable effects were found by Halpern *et al.*[36] using infrared peak shifts as criterion; bonding was shown with other chlorinated solvents but not with heptane. Clearly, for good bonding one needs not only an oxygen of enhanced negativity, but also a hydrogen of enhanced positivity.

Isomerization

(*a*) *Thiono-Thiolo Isomerization.* The early work with organophosphorus compounds was carried out without the realization that some of these compounds changed spontaneously into isomers, which had properties quite unlike their parent forms. Thus, parathion was reported to be a potent anticholinesterase;[1, 24, 34, 42a, 61, 74, 92] in 1951, Diggle and Gage[21] showed that carefully purified parathion was a very poor anticholinesterase. It is now realized that all compounds containing (I) are liable to isomerize to (II), and that this change has important effects on the properties of the compounds. This isomerization is particularly liable to occur on heating, and therefore commonly arises during distillation of such compounds.



(I)



(II)

We shall first give the basic facts about thionophosphate isomerization, and then proceed to a more detailed and documented discussion.

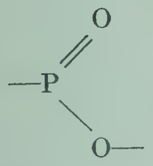
(*1*) The commonest form of isomerization is of phosphorothionates of the form $(RO)_2P(S)OX$, where R is an alkyl group, and X may have many forms. The product may be $(RO)(RS)P(O)OX$ or $(RO)_2P(O)SX$, depending upon the compound (and to a lesser extent, upon conditions).

(*2*) The configuration (I) is less stable than (II) and therefore isomerization of (I) to (II) is to be expected. Putting this more precisely, the equilibrium (I) → (II) is usually far to the right, so that an equilibrium mixture usually contains mainly (II).

(*3*) Isomerizations of the form (I) → (II) are commonly of two types. The first type is relatively slow, and occurs when there is no stable intermediate between forms (I) and (II). In these cases, relatively vigorous conditions are needed to produce extensive isomerization, e.g., heating at 150°C. or more for hours. Examples are parathion and malathion. The second type is fast, and occurs when a stable compound can be formed of a structure intermediate between that of (I) and (II). Examples are Tetram and Systox. Even when no heat has been applied, one should always suspect that samples reputed to be type (I) will be contaminated with (II), and that the contamination will increase with time.

(*4*) Isomerization is hastened by heat and light. If there is an ionic intermediate between (I) and (II) (commonly an immonium ion, R_4N^+, or a sulfonium ion, R_3S^+), isomerization will be hastened by polar solvents, the effect being proportional to the polarity of the solvent.

(*5*) Isomerization of (I) to (II) usually increases the susceptibility to hydrolysis, and increases the anticholinesterase activity. Both effects are commonly very extreme, and consequently a very small degree of isomerization can have a profound effect upon these properties. Toxicity is often changed, but not so radically, because most of the changes in properties are due to the substitution of P═O for P═S (rather than of P—S— for P—O—), and animals oxidize P═S readily to P═O; thus (I) would be oxidized to



(*6*) Isomerization of (I) to (II) increases the polarity of the compounds, and therefore (II) can be separated from (I) by appropriate paper or column chromatography.

(*7*) The simplest way to determine whether isomerization has occurred is by infrared analysis: the group P═O has a distinct absorption; the P═S group also has its own absorption in many cases. The method can also be used to determine the extent of isomerization.

Other ways of following isomerization are: (*a*) chromatographic separation followed by a determination which can be nonspecific;[29] (*b*) following the disappearance of P=S (determined by nitric acid oxidation);[62] (*c*) observing the hydrolysis rate of any given sample; and (*d*)—in the case of small samples, which are often all that are available—using the rate of change of anticholinesterase activity as index.[2] This is considered in detail below.

The first form of isomerization is that of parathion, methyl parathion, and malathion all of which isomerize to give primarily the *S*-alkyl isomer.[58, 62, 68, 93] Parathion isomerization occurs to the extent of about 50% upon heating the undiluted compound for 5 hours at 140–180°C.[65] Methyl parathion, under similar conditions, yields about 83% of the isomer; at 100°C. an ethanolic solution isomerizes 35% in 4 hours; even standing at room temperature for 5 months produces fairly substantial isomerization in an ethanolic solution.[62] It seems, then, that isomerization of this type proceeds more rapidly the smaller the alkyl group. Isomerization of methyl parathion by ultraviolet light occurs to a small extent, e.g., 1.2% in 72 hours.[62] The increase in the anticholinesterase activity of parathion upon exposure due to daylight, and even more upon exposure to ultraviolet light[71] is probably due to isomerization. Sandi,[76] on the other hand, obtained reduction of parathion on exposure to light, and this reduced form should be a very poor anticholinesterase. However, his sample was very probably highly impure, and the experiment was very crude.

The isomerization of the isopropyl analog of parathion yielded an unknown amount of what was probably an isomer, following heating of the pure crystals at 150°C. for up to 6 hours; but no isomer was produced at 110°C. (6 hours). Malathion isomerized about 90% upon heating at 150°C. for 24 hours.[62]

All the above figures are only approximate: in the first place, there are invariably other products. Metcalf and March found 7 products as well as the parent compound in a parathion isomerization mixture,[62] and the malathion isomerization mixture consists of at least two components in the chloroform-soluble fraction[67] besides parent compound and a chloroform-insoluble fraction that may contain up to 85% of the total phosphorus.[68] Considerable differences are to be found in the literature concerning both the appearance and properties of isomerization mixtures prepared in apparently similar ways (e.g., references 62 and 68), and these may be attributed to differences in starting material (particularly with respect to contaminants that may catalyze the isomerization) and procedural variations, such as the quantity treated at one time.

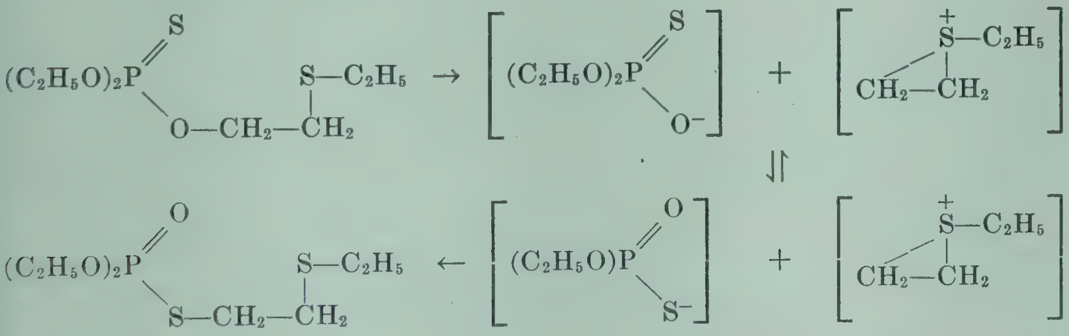
The second form of isomerizations is of the type $(RO)_2P(S)OX$ to $(RO)_2P(O)SX$, and is the common form where X is other than an aryl group [except, of course, where such a pathway is blocked in compounds of the form $(RO)_2P(S)SX$]. The phenomenon has been studied by Fukuto and

Table 2.11. Effect of Solvents and Temperature upon Systox Isomerization[a]

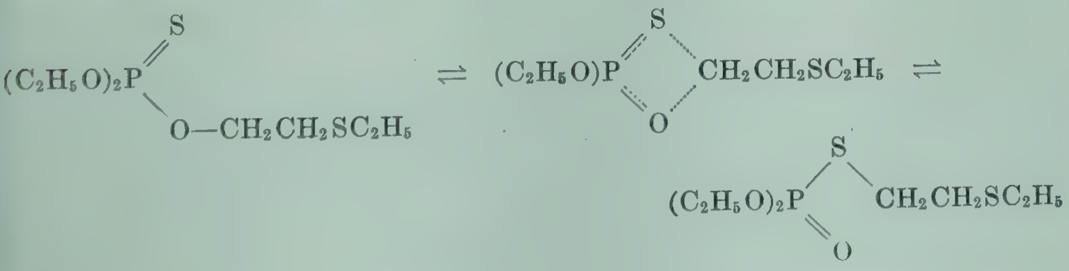
Solvent	Temperature (°C.)	k_1 sec.$^{-1}$ (rate constant of isomerization)	Isomerization % after 39 days
Ethanol	37.2	6.1×10^{-7}	56
Chloroform	37.2	8.0×10^{-8}	23.5
Methyl ethyl ketone	37.2	—	8.6
Dioxane	37.2	—	4.8
Ethyl acetate	37.2	—	4.3
Benzene	37.2	—	4.1
2,2,4-Trimethylpentane	37.2	—	1.8
None	37.2	1.8×10^{-8}	6.0
None	59.5	3.7×10^{-7}	—
None	78.2	3.7×10^{-6}	—
None	95.0	2.2×10^{-5}	—

[a] Data of Fukuto and Metcalf.[29]

Metcalf[29] in particular for Systox (which is defined as a mixture of the two isomers), using P^{32}-labeled Systox and separating the isomers by paper chromatography. The isomerization, both in the presence and in the absence of solvent, was found to conform precisely with first-order reaction kinetics. The actual rate depended upon the solvent used, and was greatly enhanced by polar solvents such as ethanol, as shown in Table 2.11. In view of these observations, it was suggested that an ionic intermediate might be formed as follows:

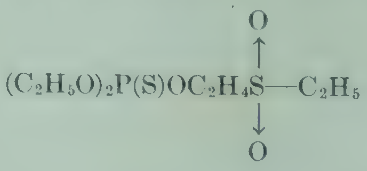


An alternative, that would, however, not explain the enhancing effect of polar solvents, was suggested:



Another careful study of this isomerization by Henglein and Schrader[43] used changes in infrared absorption to follow the isomerization. They also found that polar solvents markedly increased the isomerization rate, whereas apolar solvents decreased it. However, the quantitative results disagree rather markedly with those obtained by Fukuto and Metcalf, who demonstrate very clearly that the isomerization was first-order both for the undiluted or the dissolved compound, and that this condition held over the whole reaction range studied; a range that for the undiluted compound extended from about 20 % to about 91 % isomerization. Henglein and Schrader showed (with equally convincing graphs) that between 0 and 50 % of isomerization, the reaction was zero-order (i.e., there was a linear relationship between "time" and "% thiolo isomer") and became first-order only at higher percentages. Also Henglein and Schrader gave a t_{50} (time for 50 % isomerization) of 10 hours at 105°C. and 38 hours at 90°C.; the t_{50} calculated from the data of Fukuto and Metcalf for 95°C. (which one would expect to fall between the two figures above) is 87 hours. Henglein and Schrader commented on these discrepancies briefly, suggesting that they arise from the different analytical procedures, and felt that their infrared technique was more reliable; but the small scatter of the data of both groups, and the unambiguity of both analytical techniques, suggest that both are highly reliable. At present, the only conclusion that can be drawn is that the variables in isomerization work are such that the best workers in the most careful studies can disagree seriously. Transalkylation (discussed below) may be a factor in both studies.*

Henglein and Schrader also show that the meta-Systox, the dimethoxy analog of Systox, isomerizes in the same qualitative way as Systox itself, but more rapidly. Thus, the t_{50} at 120°C. is 2.45 hours for Systox; at 119°C. it is 0.66 hours for meta-Systox. The energy of activation for the isomerization was also lower for meta-Systox (22.8 kcal. mole^{-1}) than for Systox (25.4 kcal. mole^{-1}). They report an important confirmation of the ionic intermediate mechanism given above: the sulfone derivatives of the thiono isomers of Systox and meta-Systox do not isomerize, and this can be explained by the impossibility of such a sulfur forming an onium ion.



$$(C_2H_5O)_2P(S)OC_2H_4\overset{\overset{O}{\uparrow}}{\underset{\underset{O}{\downarrow}}{S}}—C_2H_5$$

Sulfone of Systox thiono isomer

By analogy with parathion, one might expect the thiono-form of Systox to be isomerized by light. Cook[17] has shown, however, that both Systox isomers are converted by light to new compounds, more polar than and with anticholinesterase activity similar to their parent compounds. Their identities are unknown.

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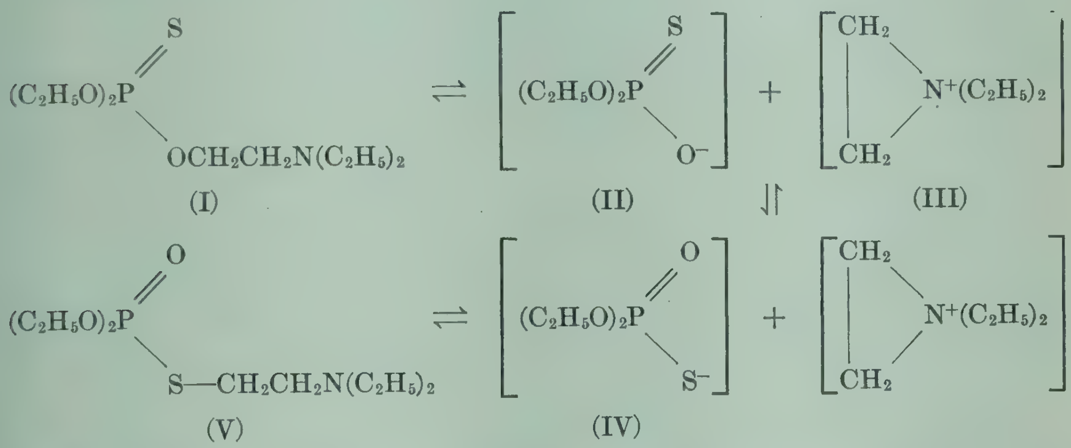
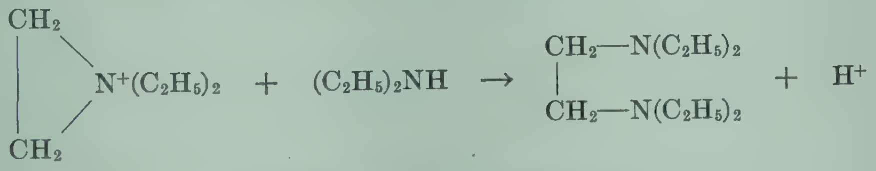
* See page 371 for a resolution of this problem.

Another case of rapid isomerization promoted by the formation of an intermediate was found with the thiono isomer of Tetram (I) in a study by Fukuto and Stafford:[32]

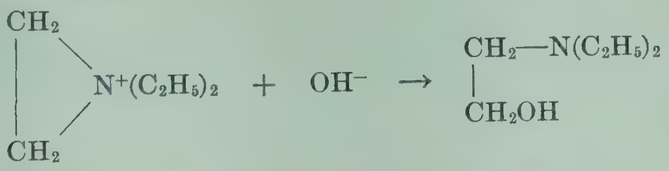


Their proof was a chemical one: they showed that solutions of (I) must be in equilibrium with (III), because:

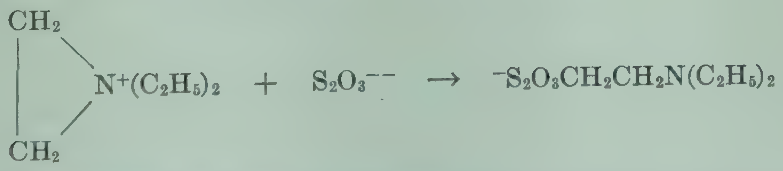
(*a*) addition of diethylamine yielded tetraethylene diamine



(*b*) alkaline treatment yielded diethylamino-ethanethiol



(*c*) an addition product was formed with thiosulfate



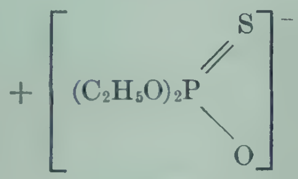
Reaction (*c*) is particularly good evidence, since thiosulfate reacts only with those compounds of this type which contain or are in equilibrium with immonium salts.

The above isomerization occurred very readily: thus at 73.8°C. it isomerized 74% in 7 hours, about ten times faster than Systox isomerization.[32]

Tammelin[83] has studied the isomerization at 100°C. of the dimethylamino analog of thiono-Tetram $(C_2H_5O)_2P(S)OCH_2CH_2N(C_2H_5)_2$ (VI). Here the expected isomerization to the $..P(O)SCH_2CH_2..$ compound was shown on infrared evidence. A zero-order type isomerization was found. Tammelin suggested an immonium ion formation which is rather more elaborate than the above picture, in that the immonium ion formed as above adds immediately to an unaltered molecule of (VI), so that:

$$(VI) \quad \rightarrow \quad [(C_2H_5O)_2P(S)OCH_2CH_2N(CH_3)_2{-}CH_2CH_2N(CH_3)_2]^+$$

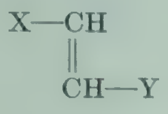
(VII)



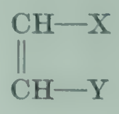
(VIII)

Then a series of other elaborate changes were proposed, but there was no supporting evidence, and the simpler scheme of Fukuto and Stafford seems much more acceptable. Tammelin also suggested that the observed zero-order kinetics might be due to (*a*) the fact that the salt form(s) may be in saturated solution; (*b*) the rate-determining step(s) is that involving reaction of the salt form(s). Consideration (*a*) is quite likely, since the isomerization was carried out with undissolved compound. But Fukuto and Stafford, working with Tetram under these conditions at 74° reported that for the first 4.25 hours the reaction was first-order; Tammelin's data were mainly for the first 5 hours, and should be comparable. Once more the paradox appears to have no simple explanation.

(*b*) *Cis-Trans Isomerism.* This kind of isomerism occurs if there is a double bond in a compound, and this double bond is not in a ring. Furthermore, each carbon of the C═C must have two different substituents on it. Under such conditions one can get for example:

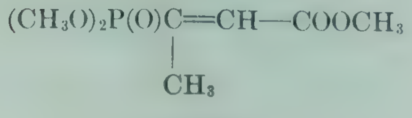


trans isomer



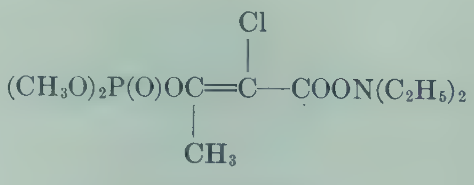
cis isomer

Such conditions are fulfilled in Phosdrin and phosphamidon.



$$(CH_3O)_2P(O)\underset{CH_3}{C}{=}CH{-}COOCH_3$$

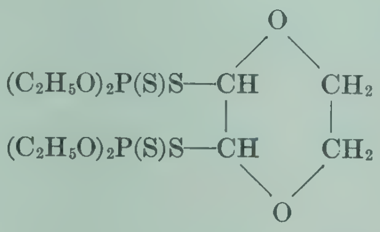
Phosdrin



$$(CH_3O)_2P(O)O\underset{CH_3}{C}{=}\overset{Cl}{C}{-}COON(C_2H_5)_2$$

Phosphamidon

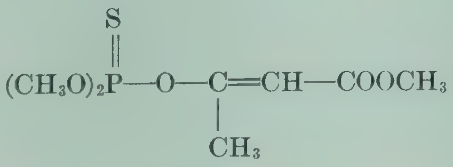
The reason for this isomerism is that the two carbons of C=C cannot rotate freely around the axis of the bond as can those in C—C. For a similar reason *cis-trans* isomerism is also found in Delnav, where rotation is restricted by the dioxane ring:



The *cis* and *trans* forms of such compounds may differ considerably in their physical properties. Thus, the *cis* isomer of Phosdrin is the less stable in alkali (half-life 28°, pH 11.6: 1.8 hours for *cis*, 3.0 for *trans*) and this is also true of the ethoxy analog of Phosdrin (half-life 3.4 hours for *cis*, 9.0 for *trans*). The *cis* isomer of Phosdrin is the less polar of the isomers, as judged by its low water-solubility and its high partition coefficient in hexane-water (5.5 for *cis*, 1.1 for *trans*) and in carbon tetrachloride-water (58 for *cis*, 14 for *trans*). Similar relations hold for the ethoxy analog.[11, 15] For Delnav, the isomers differ less: thus, the half-lives in alkali are 3.0 and 3.1 hours for *cis* and *trans*, respectively. The *trans*, is in this case, a little less polar than the *cis* isomer, as it is eluted by heptane from celite-acetonitrile a little more readily than is the *cis*.[4]

The percentage of *cis* and *trans* forms in a given mixture depends largely upon the synthesis route. Thus, one method for Phosdrin yielded over 90% of *trans*, whereas another yielded only 33% of *trans*. Casida has shown[11] that irradiation of any mixture of these isomers with ultraviolet light gave, eventually, a mixture containing about 30% *cis* form. The reaction was readily followed since the *cis* form only absorbed at 11.0 in the infrared.

Spencer found that his preparation of thiono-Phosdrin was 100% *trans* form.[79]



$$(CH_3O)_2\overset{\overset{\displaystyle S}{\|}}{P}—O—\underset{\underset{\displaystyle CH_3}{|}}{C}=CH—COOCH_3$$

Thiono-Phosdrin

After irradiation of a carbon tetrachloride solution of this for 30 hours with ultraviolet light, about 50% of *cis* was produced, with negligible thiono-thiolo isomerization occurring. The *cis* and *trans* forms were separated using an alumina column, from which carbon tetrachloride eluted only the relatively apolar *cis* form.

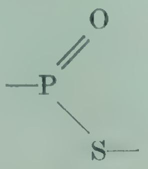
(*c*) *Methodology.* Finally, a comment upon kinetic analysis of hydrolysis data as a means of studying the purity of a compound.

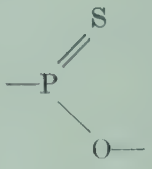
Although side-reactions can and do occur, the hydrolysis of a pure organophosphate usually follows first-order kinetics, i.e., a plot of log concentration of remaining compound against time will yield a straight line. This is taken to be due to conformity of the reaction with the first-order rate equation

$$k = \frac{2.303}{t} \log \frac{a}{a-x}$$

where k is a constant (the first-order rate constant), a is the initial concentration of the compound, and x is the amount hydrolyzed at time t.

Aldridge and Davison[2] carried out kinetic studies on pure and impure samples of diethyl *p*-chlorophenyl phosphate. In this case isomerization is not a problem, but the principles they used can be applied directly to the case of isomerization mixtures, since form (I) is invariably much more hydrolyzable than form (II).


(I)


(II)

Hydrolysis was followed by studying the rate of loss of anticholinesterase activity, since hydrolysis products of organophosphates (if the products are anionic) are invariably ineffective as anticholinesterases. Figure 2.4 shows two curves: the top one is that given by "purified" diethyl *p*-chlorophenyl phosphate, i.e., a compound that had been allowed to stand 23 hours in buffer in order to decompose unstable compounds. The other has two intersecting lines, showing the presence of one unstable compound which has almost disappeared in 30 minutes, and one more stable compound, presumably the diethyl *p*-chlorophenyl phosphate itself. From such data it is possible to compute the percentage of impure material present; but one must have some independent measure of the anticholinesterase activity per mole of the components. In principle, such studies can be extended to more than two components, so long as the components have adequately different hydrolysis rates.

A practical difficulty may arise in the application of such a method to isomerization mixtures: the P═S form may be too ineffective an anticholinesterase to give detectable enzyme inhibition at convenient concentrations. One would then get a plot due entirely to the antienzymic isomer (assuming that there was only one isomeric product). In such cases a titration of acid liberated during alkaline hydrolysis might be a better index. This

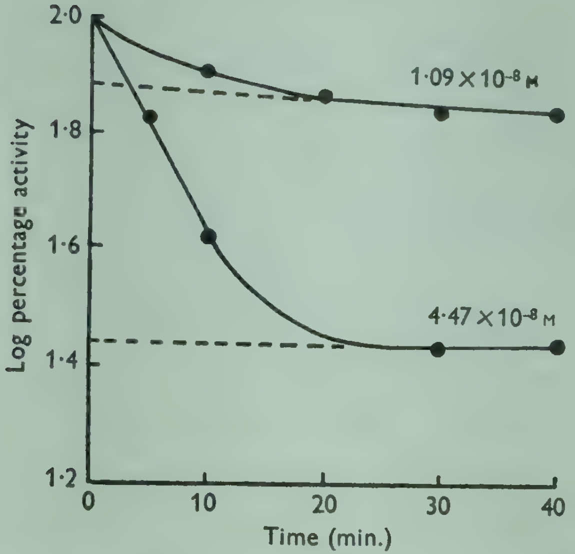


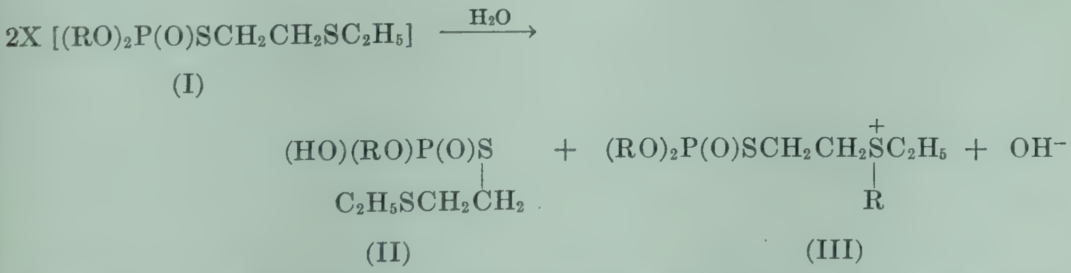
FIG. 2.4. Rate of inhibition of erythrocyte cholinesterase by 2.7×10^{-8} *M* TEPP plus 4.0×10^{-5} *M* diethyl *p*-chlorophenyl phosphate, at zero time and after 23 hours in bicarbonate buffer at room temperature. From Aldridge and Davison.[2]

method has been used in studying mixtures of closely related organophosphates which varied in their hydrolyzabilities.[78]

Transalkylation

Heath and Vandekar[42] have made a very thorough study of the reactions that take place when Systox type compounds are stored, either in aqueous solutions or undiluted. The importance of these phenomena is exemplified by their observation that a carefully purified sample of the thiolo isomer of methyl Systox, in 1 % solution, changed during the course of 1 day at 35°C. so that its LD_{50} to the rat fell from 60 to 2 mg./kg.; a solution of an impure sample of thiono isomer changed in 1 hour at 37°C. so that its LD_{50} fell from 220 to 2 mg./kg.

Two principal reactions appear to be involved in this phenomenon of increasing toxicity. One, as shown by methyl and ethyl thiolo-Systox, is a self alkylation.



$$2X\ [(RO)_2P(O)SCH_2CH_2SC_2H_5] \xrightarrow{H_2O}$$

(I)

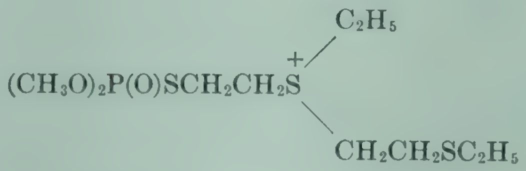
$$(HO)(RO)P(O)\underset{\displaystyle C_2H_5SCH_2CH_2}{S} + (RO)_2P(O)SCH_2CH_2\overset{+}{\underset{\displaystyle R}{S}}C_2H_5 + OH^-$$

(II) (III)

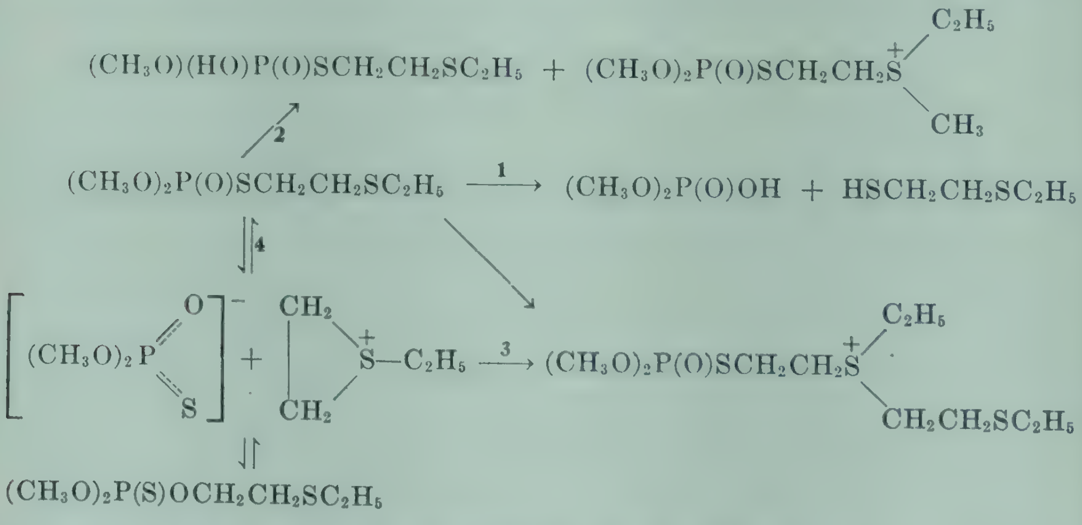
Compound (III) is cationic, as demonstrated electrophoretically, and when R is a methyl group, it is about 1000 times as toxic to the rat as (I). The reaction occurs in stored samples and in aqueous solutions; in the latter case, normal hydrolysis occurs too. The transalkylation reaction is rapid when R=CH_3, slower for R=C_2H_5. In the methyl compound, the thiolo isomer of (I) appears to be in dynamic equilibrium with the thiono; and the thiono can undergo another spontaneous reaction which makes it a more potent anticholinesterase. This more potent derivative may be the self alkylated form:

$$(CH_3O)_2P(S)OCH_2CH_2\overset{+}{S}(CH_3)C_2H_5$$

Alternatively, it may be formed from a reaction of the thiolo isomer with the thiolo-thiono transition complex (see above under "isomerization"), to produce:



If we accept this possibility, we can now show the reactions undergone in aqueous solution, to yield 7 more phosphorus-containing products:

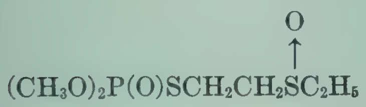
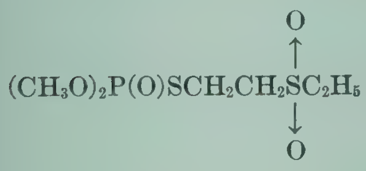
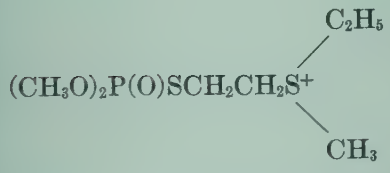
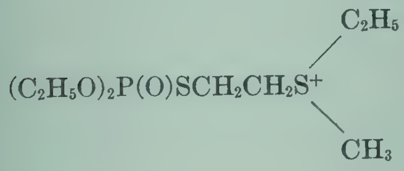
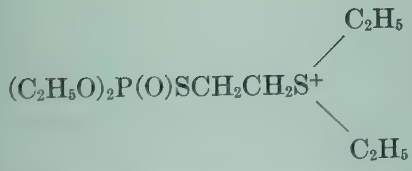


Reaction 2 is not directly undergone by the sulfoxide derivative, i.e., $(CH_3O)_2P(O)SCH_2CH_2S(O)C_2H_5$, but a certain amount of reduction of the —S(O) to —S— occurs (followed by transalkylation as above), with simultaneous oxidation of another molecule: probably not by S(O) to —$S(O_2)$— but by an oxidation of a hydrolysis product, e.g., $(CH_3O)_2\cdot P(O)SH$.

The sulfone, $(CH_3O)_2P(O)SCH_2CH_2S(O_2)C_2H_5$, is quite stable; the pure compound does not yield toxic products even on heating.

The readiness with which these reactions occur give a severe warning to those who would work with them, and the methodological section of

TABLE 2.12. PROPERTIES OF SYSTOX-TYPE COMPOUNDS AND THEIR DERIVATIVES[a]

Compound	LD50 (rat) (mg./kg.)		I_{50} (M)
	Intravenous	Oral	Sheep red cell cholinesterase
$(CH_3O)_2P(O)SCH_2CH_2SC_2H_5$	65	63	6.5×10^{-5}
 $(CH_3O)_2P(O)SCH_2CH_2\overset{\overset{O}{\uparrow}}{S}C_2H_5$	47	65	4.1×10^{-5}
 $(CH_3O)_2P(O)SCH_2CH_2\overset{\overset{O}{\uparrow}}{\underset{\underset{O}{\downarrow}}{S}}C_2H_5$	22	32	2.3×10^{-5}
	0.06	9.8	3.9×10^{-8}
$(C_2H_5O)_2P(O)SCH_2CH_2SC_2H_5$	—	—	5.4×10^{-6}
	0.016	20	4.7×10^{-9}
	0.010	—	2.6×10^{-9}

[a] Data of Heath and Vandekar.[42]

Heath and Vandekar's paper deserves special attention. It reveals the great care needed to obtain compounds of acceptable purity. The properties of the products of transalkylation are shown in Table 2.12.

Oxidation

There are three substituents in the common organophosphates that may be readily oxidized: thiono sulfur, mercapto sulfur, and phosphoramides. The chemistry of these oxidations will not be discussed at length (unless the products are uncertain), as this aspect is largely a matter of synthesis, a topic not considered extensively in this book. However, some information on oxidation may throw light on the biological oxidations, which are among the most important of the biological reactions of these compounds.

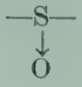
The oxidation of $(RO)_2P(S)X$ to $(RO)_2P(O)X$ is an important one, as the discussions on hydrolyzability and anticholinesterase activity show. Co-ral,[52] ronnel,[72] and malathion[84] have been oxidized by peracetic acid in anhydrous chloroform. Concentrated nitric acid has been used for malathion[45, 69] and ronnel,[72] but the method is too variable, too drastic, and often too extensive. For small-scale oxidations in solution, bromine-water is very useful for numerous phosphorothionates and remarkably simple,[26] but has been found unsuitable for preparation of the pure product, in the case of malathion.[84] *N*-Bromosuccinimide is a suitable oxidizing agent for chromatogram-scale oxidations.[18]

The spontaneous oxidation of a phosphorothionate to a phosphate has been demonstrated in the case of dimethoate[20] on the outside of leaves of potato, corn, and cotton, and also on glass plates.

$$(CH_3O)_2P(S)SCH_2C(O)NHCH_3 \rightarrow (CH_3O)_2P(O)SCH_2C(O)NHCH_3$$

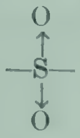
Dimethoate

The oxidation of the mercapto sulfur of compounds such as Systox (either isomer) has been accomplished with hydrogen peroxide, to yield the sulfoxide or with potassium permanganate, to yield the sulfone.[31]



$$-\underset{\underset{\textstyle O}{\downarrow}}{S}-$$

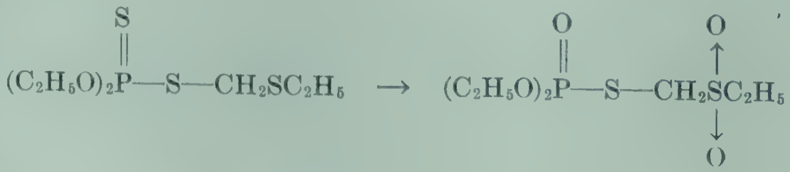
Sulfoxide



$$-\overset{\overset{\textstyle O}{\uparrow}}{\underset{\underset{\textstyle O}{\downarrow}}{S}}-$$

Sulfone

Thimet can be oxidized either to the sulfoxide or to the sulfone by controlled addition of monoperphthalic acid[8] or perbenzoic acid.[59a] Hydrogen peroxide or peracetic acid give the sulfoxide, according to Bowman and Casida,[8] although Miskus and Hassan[64] found oxidation as far as the phosphorothiolate sulfone:

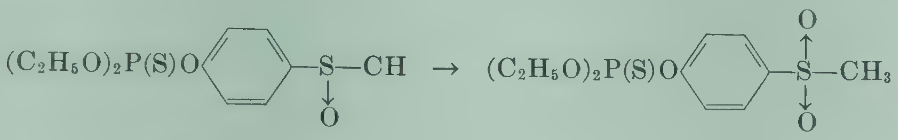


$$(C_2H_5O)_2\overset{\overset{\textstyle S}{\|}}{P}-S-CH_2SC_2H_5 \rightarrow (C_2H_5O)_2\overset{\overset{\textstyle O}{\|}}{P}-S-CH_2\overset{\overset{\textstyle O}{\uparrow}}{\underset{\underset{\textstyle O}{\downarrow}}{S}}C_2H_5$$

Thimet

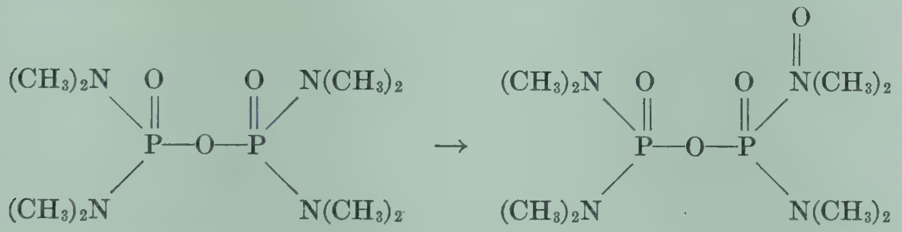
But judging from the reported data, oxidations were in all cases incomplete, and probably mixtures resulted.

The spontaneous oxidation of the sulfoxide Bayer 25141 to its sulfone has been reported,[6] following exposure of thin layers upon glass plates or cotton leaves.



Bayer 25141

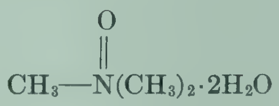
The matter of phosphoramidate oxidation is a controversial one. Most of the work has been done with schradan, one or more of whose dimethylamide groups may be oxidized. Neutral permanganate oxidation yields several products, one of which is identical with the potent anticholinesterase metabolite of schradan produced by mammals and plants,[13, 14] and insects.[70] Let us call this particular oxidation product (I). In an early discussion of the possible nature of (I), Hartley[37] proposed that it contained the *N*-oxide of one of the amide groups:



This structure has since been supported by Casida and his co-workers,[12, 13, 85] on the following evidence: (*a*) the mixed permaganate oxidation products from schradan showed an infrared absorption peak at 1681 cm.$^{-1}$ which is lacking in schradan. Trimethylamine *N*-oxide dihydrate has a peak at 1660 cm.$^{-1}$ which is lacking in trimethylamine:

$CH_3—N(CH_3)_2$

Trimethylamine

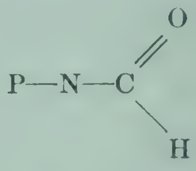


$$CH_3—\overset{\overset{O}{\|}}{N}(CH_3)_2\cdot 2H_2O$$

Trimethylamine *N*-oxide dihydrate

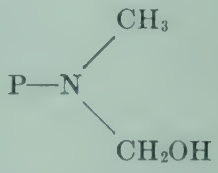
(*b*) under acid condition, (I) reacts to produce formaldehyde; (*c*) partitioning data suggest that (I) is more basic than schradan (the data were later considered doubtful;[85] (*d*) (I) is about 5 × 10^5 times better as an inhibitor of cholinesterase than is schradan; (*e*) (I) can isomerize to form a poor anticholinesterase.

Of these data, only (*a*), above, clearly suggests an *N*-oxide. But it has now been shown[80] that the material with a 1681 cm.$^{-1}$ peak is not (I), since the "1681 compound" may be separated from the anticholinesterase compound in the Craig counter-current apparatus. The material absorbing at 1681 $cm.^{-1}$, which we shall call (II), is very much more stable and less polar than (I), and does not yield formaldehyde when treated with acid. (II) might contain the

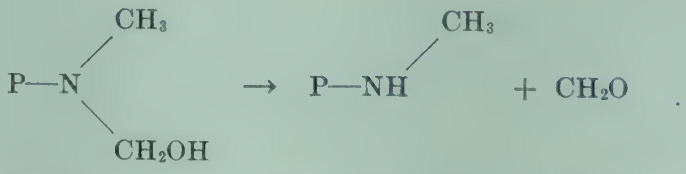


structure. It has also been shown[80] that although trimethylamine *N*-oxide dihydrate absorbs (weakly) at 1660 $cm.^{-1}$, the anhydrous oxide has no such absorption; therefore, the absorption is probably not due to N → O. This is confirmed by the fact that anhydrous dimethyltryptamine *N*-oxide does not absorb between 1600 and 1700 $cm.^{-1}$.

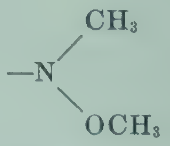
What, then, is the structure of (I)? Heath *et al.* have pointed out that its partition coefficient in comparison to schradan itself conforms with its containing the grouping[41]



and so we may call it hydroxymethyl schradan. Its reactions include (*a*) decomposition to heptamethylpyrophosphoramide with formaldehyde release:



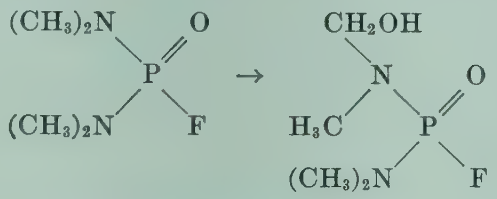
(*b*) hydrolysis at the P—O—P bond, which occurs readily (e.g., half-life at 25°, pH 8 = 42 minutes); (*c*) isomerization to (III). This isomerization is catalyzed by acid, alkali, and heat. Tsuyuki *et al.*[85] proposed the following substituent for (III),



by analogy with certain amine oxide rearrangements. Later, Spencer *et al.*[80] synthesized this proposed compound, and found that it partitioned quite differently from (III). The structure of (III) is therefore unknown; the substituent P—O—N$(CH_3)_2$ has been mentioned as a possibility.[85]

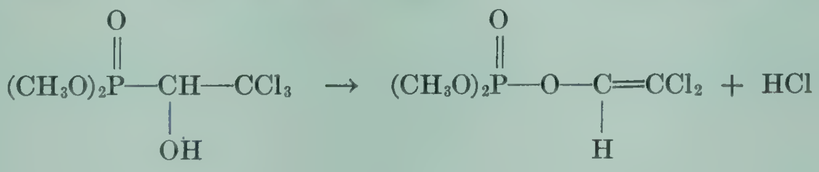
Schradan can also be oxidized to (I) by sodium hypochlorite, and less effectively by potassium dichromate, bromine water, and hydrogen peroxide.[85] However, peracetic acid oxidation yields (III). The fact that peracetic acid oxidation gives a product which is a poor anticholinesterase [and is therefore not (I)] but absorbs[85] at 1681 cm.$^{-1}$ is a further demonstration that the 1681 cm.$^{-1}$ peak is not caused by (I).

Dimefox can also be oxidized by peracetic acid, permanganate, and peroxide, and the picture of the concurrent appearance of new infrared absorbing components and anticholinesterase components appears very similar to that of schradan.[3] There seems to be no reason to doubt that the derivatives thereby produced are analogous to those produced by oxidation of schradan:



Dehydrohalogenation

While conducting studies upon Dipterex, Mattson *et al.*[59] observed that a very toxic volatile impurity was present which could be obtained, also, by preparing the contaminated Dipterex in ether and washing out the parent compound with water. Later they found that alkali treatment of aqueous Dipterex solutions gave a large yield of the same toxic impurity in the form of an oil that separated out. These workers proposed various structures for the impurity, but Barthel *et al.*[5] first demonstrated that the impurity was DDVP, produced by the unexpected rearrangement:

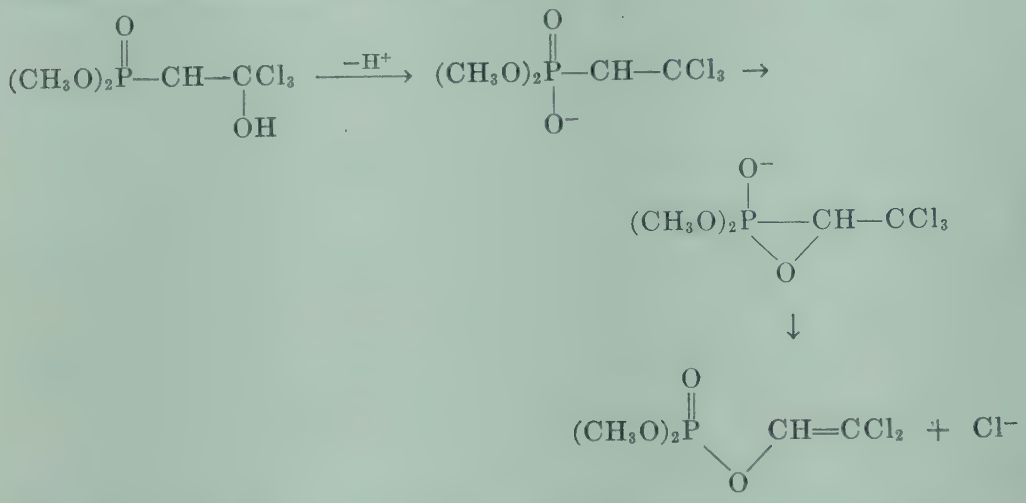


$$(CH_3O)_2\overset{O}{\overset{\|}{P}}-\underset{OH}{\underset{|}{CH}}-CCl_3 \rightarrow (CH_3O)_2\overset{O}{\overset{\|}{P}}-O-\underset{H}{\underset{|}{C}}=CCl_2 + HCl$$

Dipterex DDVP

Bartel, *et al.* proved the formula by synthesis of DDVP and the other possible compounds. Almost simultaneously, Lorenz *et al.*[57] arguing on the basis of the infrared absorption, demonstrated that DDVP was the only feasible product. DDVP is now used as an insecticide itself. The unresolved question as to whether Dipterex owes its biological activity to DDVP formation will be considered later.

The dehydrochlorination of Dipterex was studied by Metcalf, *et al.*[60] It was strongly pH-dependent, being negligible at pH 5.4, and 60% complete in 2 hours at pH 8. The reaction followed first-order kinetics only initially. Under anhydrous conditions, bases (pyridine, triethylamine) induced the reaction. The mechanism depending upon proton removal, first suggested by Kharasch and Bengelsdorf,[49] was therefore accepted:



This mechanism satisfactorily accounts for both the aqueous and anhydrous dehydrochlorination.

Effects of Light

Sandi[76] reported that parathion was reduced by sunlight, presumably giving diethyl *p*-aminophenyl phosphorothionate. His techniques and material were both crude, however, and the reaction requires substantiation.

Usually, both visible and ultraviolet light cause reactions which produce compounds more potent than their parents in inhibiting cholinesterase. This has been attributed to an acceleration of isomerization, e.g., for methyl parathion[62] and parathion.[71] In the case of Systox isomers; Cook[17] has shown that both are altered following exposure to light, producing new compounds, which he separated chromatographically and whose infrared spectra he studied. The thiono isomer, which was changed to a compound which contained the P=S group, was not a strong anticholinesterase, and was more polar than the parent compound. The thiolo isomer was changed analogously, giving a P=O-containing compound which (like its parent) was a strong anticholinesterase, but which was more polar than the parent. Under similar conditions, the following compounds were not affected: parathion, methyl parathion, Chlorthion, malathion, EPN and Diazinon.

The nature of the products is unknown. Under the experimental conditions, one might expect substantial transalkylation; and a photo-induced oxidation of the mercapto-sulfur to a sulfoxide or a sulfone is feasible. However, these reactions would lead to more potent anticholinesterases, and this does not appear to have occurred (although the data is not conclusive on this point).

In another study, Cook[18] showed that exposure of numerous organophosphates to ultraviolet light led in every case to the production of new potent anticholinesterases which were more polar than the parents. This was not only true of the 9 phosphorothionates, but also of schradan, TEPP, and Dipterex. No clue was given as to structure. A rather more extensive study of parathion irradiation[27] showed that as the irradiation time increased, the following changes occurred progressively: (*1*) an increase in anticholinesterase activity; (*2*) a decrease in the toxicity to the house fly; (*3*) a decrease in the material assayed by the Averell-Norris technique, i.e., nitro-aromatic compounds; (*4*) a decrease in the ability to lower the blood cholinesterase of rats following chronic feeding of the material.

The nature of the products is "undoubtedly . . . a mixture of parathion, paraoxon, and other oxidation and degradation products,"[27] and isomers of parathion may also be expected.

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CHAPTER 3

The Reaction with Cholinesterase *in Vitro*

It will be shown in later chapters (6 and 7) that the toxic effects of organophosphates are to a great extent due to their ability to inhibit acetylcholinesterase. In this chapter, this process of inhibition will be described under the relatively simple conditions of reaction in free solution and the absence of interfering enzymes (such as those that can decompose organophosphates).

For these simple conditions, there are three principle reactions involved in enzyme-phosphate interaction. Using, as before, the type formula $(RO)_2P(O)X$, where R is an alkyl group, usually methyl, ethyl or isopropyl, and X is an acidic residue such as F, *p*-nitrophenol, or another $(RO)_2P(O)O$ group, these reactions are as follows:

(*a*) *Phosphorylation of the Enzyme.* The rate of this reaction depends upon (1) the excellence of "fit" of the inhibitior on the enzyme surface, which is assisted by an appropriately placed cation; (2) the ability to phosphorylate a serine (or perhaps a histidine) in the enzyme active site; the nature of X in compounds $(RO)_2P(O)OX$ is a major factor here. The speed of all subsequent reactions can only depend upon the nature of R, for X is removed in phosphorylation, and only $(RO)_2P(O)$-enzyme remains.

(*b*) *Reversal of Inhibition.* This reaction is particularly fast when R is methyl. Very many nucleophilic reagents speed up this reaction enormously.

(*c*) *Aging.* That is, the formation of a fully irreversible form of the enzyme. The speed of this process depends upon the nature of R, but for different cholinesterases the optimal R groups are different.

Phosphorylation of the Enzyme

The most striking and important property of many organophosphates is their capacity at very low concentrations to combine with cholinesterase. All the potent organophosphates (or their oxidized forms) can inhibit one or other cholinesterase by 50% or more at a concentration of 10^{-6} *M*. The majority are effective at between 10^{-6} and 10^{-9} *M*. A good measurable cholinesterase concentration would be 10^{-11} *M* with respect to active centers*; consequently the phosphates are in great excess. But there is also a very great number of phosphorylatable amino acids not associated with the centers, particularly in crude cholinesterase preparations,[56, 63] and also in relatively pure ones.[30]

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* Thus, a cholinesterase preparation releasing 224 μl. of CO_2 in 30 minutes from a bicarbonate buffer hydrolyzes 2×10^{17} molecules of acetylcholine per minute. The turnover number is about 5×10^5,[28] so the concentration of active sites is about 2×10^{-11} *M*. Much higher enzyme concentrations are often used, however: Jandorf *et al.*[64] uses levels around 10^{-6} *M*, Nachmansohn *et al.*[76] from 10^{-7} to 10^{-11}.

The organophosphates all act by combining with the esteratic site of cholinesterase in an "irreversible" manner. The significance of the quotation marks will become apparent later. For the moment, let us say that except in the presence of special agents, the reaction is in the majority of cases irreversible in the normal course of an experiment (e.g., up to 4 or 5 hours). In this way these compounds differ sharply from the carbamate inhibitors (of which the best known is eserine) which react reversibly; thus a given eserine-inhibited sample of cholinesterase may have its inhibition lessened or removed by dialysis, dilution, or (if the enzyme is particulate) by washing.[3, 6, 21, 70] Organophosphate-inhibited cholinesterase is unaffected by these procedures if carried out rapidly or in the cold.[3, 21, 71]

It is now firmly established that this combination of organophosphate with cholinesterase involves not merely an additive binding but an actual phosphorylation, so that inhibition by a diethyl phosphate leads to diethyl phosphorylated cholinesterase. The most direct proof is that the nature of the inhibited cholinesterase—as judged by its rate of recovery from inhibition—is characteristic only of the nature of the alkoxy phosphate moiety of the inhibitor. Thus, a single kind of inhibited cholinesterase is given by: dimethyl *p*-nitrophenyl phosphate, dimethyl phosphorofluoridate, dimethyl phosphoric anhydride and *O,O*-dimethyl *S*-*p*-nitrophenyl phosphorothiolate[9] (Fig. 3.1). The reaction may be represented (when EH is uninhibited cholinesterase) as:

$$\mathrm{EH} + (\mathrm{RO}_2)\mathrm{P(O)X} \rightarrow (\mathrm{RO})_2\mathrm{P(O)E} + \mathrm{XH}$$

Secondly, the energy of activation of the inhibitory reaction is in the correct range for a chemical reaction, e.g., 10.6 kcal. mole^{-1} for paraoxon against erythrocyte cholinesterase[5] and 14.5 kcal. mole^{-1} for *N,N′*-diisopropyl phosphorodiamidic anhydride against rat jejunum pseudocholinesterase.[34]

An indirect proof comes from work on another esterase, chymotrypsin. This esterase has the advantage over cholinesterase in that it may be crystallized and its molecular weight obtained with accuracy. It was shown for this esterase that when organophosphates $\mathrm{R_2P(O)X}$ react with it, one mole of HX appears for every active site that is inhibited. This has been shown for DPF,[66] for TEPP,[36] and for paraoxon.[54] With electric eel cholinesterase, Michel and Krop have shown that labeled DFP is bound so firmly to the enzyme that it does not dissociate in 10% trichloroacetic acid,[74] and with human plasma cholinesterase, Brauer and Pessotti[22] showed that labeled "HETP" (usually considered, nowadays, to owe its activity to its TEPP content) is bound so firmly that it is not removed by acetone precipitation of the enzyme. Brauer's early work[21] had shown no binding of HETP[32] to alochol-precipitated cholinesterase but this was probably a result of inadequate labeling of the TEPP fraction. Boursnell and Webb[20] previously showed that labeled DFP was bound to horse serum cholinesterase and liver esterase, and was not removed by acetone precipitation; more important, it was not bound to cholinesterase that had been heat-denatured, and yet when bound to active cholinesterase it was not removed by subsequent heat denaturation.

The fact that the inhibition of both true and pseudocholinesterase follows the kinetics normally found for a two component reaction, one component being in excess, is further strong evidence that a reaction with the enzyme occurs. This important aspect is expanded below.

Another piece of evidence[96] is the analogy between acetylation of cholinesterase by acetylcholine (which of course is rapidly terminated by hydrolysis of the acetylated enzyme) and phosphorylation by the phosphates. In both cases the esteratic site and an X═O group are involved; in both cases there is a pH optimum between 7 and 9 which is due to changes in the enzyme site with pH.

By now, a great number of pieces of supporting evidence for the phosphorylation exist. It will be seen, later, that not only is the spontaneous recovery rate of cholinesterase characteristic only of the alkyl substituents of the inhibitor, but so, also, is the reversibility obtained with special agents, and the "aging" rate.

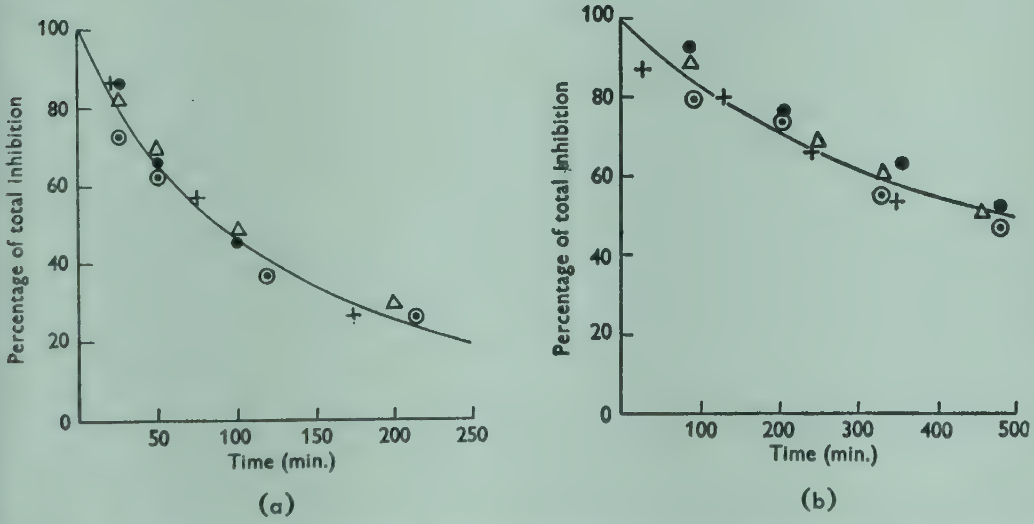

FIG. 3.1. Rate of reactivation of phosphorylated cholinesterase. (*a*) Erythrocyte cholinesterase with dimethyl phosphates: +, dimethyl *p*-nitrophenyl phosphate; ●, dimethyl phosphoric anhydride; ◉, dimethyl phosphorofluoridate; △, *O*,*O*-dimethyl *S*-*p*-nitrophenyl phosphorothiolate. (*b*) Serum cholinesterase with diethyl phosphates: +, paraoxon; ●, TEPP; △, diethly phosphorofluoridate; ◉, parathion, *S*-phenyl isomer. From Aldridge and Davison.[9]

(*a*) *Kinetics.* It was pointed out by Aldridge[3] that the reaction between inhibitor and enzyme follows the kinetics of a simple bimolecular reaction when one component (the inhibitor) is in considerable excess. Consequently, the progress of the reaction with a given concentration of inhibitor may be represented by the first-order rate equation familiar in such problems as the hydrolysis of esters:

$$k_1 = \frac{1}{t} \log_n \left(\frac{a}{a - x} \right)$$

Where k_1 is the reaction rate constant, a is the initial activity of the enzyme, and x is the activity of the enzyme after incubation for t minutes with a given concentration of inhibitor. Since the rate is directly proportional to the inhibitor concentration I,

$$k_1 = k_2 I$$

$$k_2 = \frac{k_1}{I} = \frac{1}{tI} \log_n \left(\frac{a}{a - x} \right)$$

k_2 is the bimolecular rate constant.* The ratio $a/a - x$ is $1/f$, where f is the fraction of enzyme activity remaining at time t, or $100/P$, where P is the % activity at time t. The equation may therefore be written:

$$k_2 = \frac{2.303}{tI} \log_{10} \left(\frac{100}{P} \right)$$

For ease of plotting, the equation may be rearranged:

$$\log_{10} P = 2 - \left(\frac{k_2 I}{2.303}\, t \right)$$

Thus, a plot of $\log_{10} P$ against t should give a straight line with a slope of $-k_2 I/2.303$ and a y intercept of 2. A series of such curves for different concentrations of diethyl *p*-chlorophenyl phosphate is shown in Fig. 3.2.

These kinetic findings have been amply confirmed for many inhibitors and both true and pseudocholinesterase.[3, 5, 7, 8, 34]

If, from the above equation, a plot of P versus $\log_{10} I$ is made, an unsymmetrical sigmoid results, a familiar form to those who have studied the relationship between inhibitor concentration and cholinesterase inhibition (e.g., ref.[70]). An estimate of k may, of course, be calculated from any value of P at a given t; a good value should be obtained by determining P for a number of values of t and I.

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* In the literature, one often finds the symbols K_1 and k_1, or K_2 and k_2, used interchangeably. Usually it is quite evident when a rate constant is implied. However, it would avoid possible uncertainties if authors would use k for rate constants (i.e., kinetic values) and K for equilibrium constants (i.e., thermodynamic values).

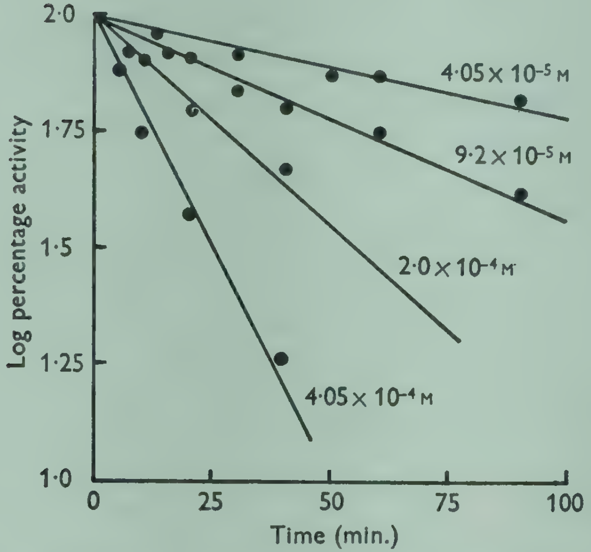


FIG. 3.2. Rate of inhibition of erythrocyte cholinesterase by purified diethyl *p*-chlorophenyl phosphate. From Aldridge and Davison.[7]

It follows from the considerations above, that the reaction is progressive, i.e., the longer the inhibitor is in contact with the enzyme, the greater the inhibition.[70, 76] Theoretically, even the weakest anticholinesterase of this type would inhibit cholinesterase 100% if left in contact long enough. (In practice, this is seldom true,[76] because of nonspecific phosphorylation, e.g., of enzymically unimportant amino acids, that utilize the organophosphate). The molar concentration of inhibitor that gives 50% enzyme inhibition is called the I_{50}. This parameter, and also the so-called pI_{50} (= the negative logarithm, to base 10, of the I_{50}) is time-dependent, and therefore is not definitive unless the incubation time is known. The correct parameter to use in stating the "strength" of an inhibitor (i.e., its affinity for cholinesterase active centers) is the bimolecular rate constant k_2 at a fixed temperature and pH. The I_{50} is far more popular in the literature, however, probably because its units (i.e., molar concentration) are readily pictured and mentally manipulated, whereas the units of k_2, the bimolecular rate constant (i.e., minutes^{-1} liter moles^{-1}), confuse all but the hardiest chemist. In general, the incubation times reported in the literature are not tremendously different, commonly being in the region of 1 hour, as this allows the whole run of preparation, incubation, and assay to be carried out in a morning or afternoon.

There have been a number of cases reported in the literature of organophosphates whose reaction with cholinesterase does not conform with first-order kinetics. There are at least six possible reasons: (1) the "compound" may contain one or more anticholinesterase impurities. The case of one impurity has been excellently dealt with by Aldridge and Davison,[7] who show how by a simple analysis one may obtain the k_2 values for both compounds; (*2*) the enzyme source may be impure, containing other reactable enzymes, or else materials which may compete at the active center; (*3*) the inhibitor, if extremely potent, may under the experimental condition not be in great molar excess over the cholinesterase active centers. This case is discussed below (page 79); (*4*) at the other extreme, compounds (such as the phosphorothionates) which are very poor anticholinesterases, and are usually studied because of the potency of their metabolic products, may not show first-order kinetics (e.g., malathion[78]). Probably the low affinity for the active centers increases the importance of nonspecific phosphorylation of other amino acid residues in the enzyme protein; (*5*) phosphorylated cholinesterase may be reversed by spontaneous reactivation (see below); (*6*) the inhibitor may be unstable in water or hydrolyzed by enzymes in the preparation.

The use of the term I_{50} has been objected to by Jandorf *et al.*[64], who say that "under ideal conditions, the commonly calculated I_{50} . . . is simply one-half the enzyme concentration, and any deviations from this relationship are due to submaximal inhibition or to side reactions such as hydrolysis of the inhibitor." But such "ideal conditions" imply exactly mole-for-mole reaction of inhibitor and enzyme, with extremely long incubations and no nonspecific phosphorylations. Furthermore, whether one finally expresses one's data as I_{50} or as k_2 value, one necessarily chooses conditions of submaximal inhibition and of excess inhibitor. So long as one does this, it is strictly true that the I_{50} at a given incubation time is as exact a measure of inhibitory potency as the k_2 value. In fact the two terms are interconvertible, since

$$\begin{aligned} k_2 &= \frac{2.303}{It} \log_{10} \left(\frac{100}{P}\right) \\ &= 0.695/I_{50}t \end{aligned}$$

where t is the incubation time. There is, therefore, no inherently greater accuracy in a k_2 value than in an I_{50} ; either may be accurately or badly determined. The advantage of a k_2 value is that it is time-independent; it is a more condensed statement. But it implies that pseudo-first-order kinetics are obeyed; and since this is not always the case, is not always applicable. Clearly, the term I_{50} has a useful place: it will be used often in this book, as will its negative logarithm, the pI_{50} .

An important implication of the conformity with first-order kinetics—indeed, a kind of restatement of the conformity—is that the extent of inhibition is independent of enzyme concentration. This is of considerable practical importance, because enzyme concentrations are difficult to establish, and may vary considerably from one technique to another (e.g., low in manometric but high in electrometric procedures). In spite of these

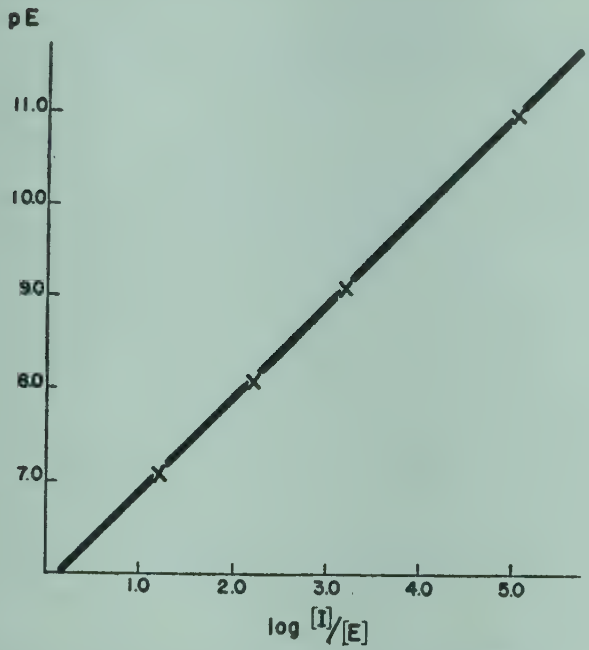


FIG. 3.3. Variation of $[I]/[E]$ with pE, where $[I]$ is DFP concentration for 50% inhibition of electric eel cholinesterase, $[E]$ is enzyme concentration, pE is negative logarithm of enzyme concentration. From Nachmansohn *et al.*[76]

variations, then, all techniques should give the same value for the reaction constant.

Nachmansohn *et al.*[76] pointed out in 1948 that the excess of inhibitor over enzyme required to give a fixed inhibition (with a given incubation time) increased with increasing enzyme concentration. In one experiment (Fig. 3.3) they studied how $\log I/E$ varied with $\log E$, for a given inhibition, and found a straight line and, thus, that "whereas the excess of molecules of inhibitor over molecules of enzyme is 25 times in the highest enzyme concentration used, more than 100,000 molecules of inhibitor are necessary for each molecule of enzyme in the lowest concentration tested in order to obtain the 50% inactivation." The implication is, of course, that I is independent of E in this experiment, i.e., that the amount of inhibitor for any given inhibition is independent of enzyme concentration. In other words, their results state that this reaction is first-order. The figure shows that first-order conditions still hold when the ratio of inhibitor to enzyme is as low as 25:1.

The above discussion applies only if the inhibitor concentration is in substantial excess of the enzyme concentration. When this condition is not obtained, the more complex second-order reaction kinetics[47] will be needed to describe the way in which inhibition varies with time and concentration:

$$k_2 = \frac{2.303}{t(a-b)} \log_{10} \frac{b(a-x)}{a(b-x)}$$

where a is the initial inhibitor concentration, b the initial enzyme concentration, and x the amount of enzyme inhibited at time t.

A rearrangement of this equation shows that plotting t against $\log_{10} (a - x)/(b - x)$ should give a straight line of slope $2.303/k(a - b)$. In such cases, then, one must know the molar concentration of enzyme in order to estimate the second-order rate constant.

Examples of this type of kinetics could undoubtedly occur with inhibitors with high pI_{50}'s (e.g., around 10), particularly if the cholinesterase concentration is high. Probably all that can be done routinely is to ensure that when such a pI_{50} is quoted, the conditions given should include some statement of enzyme level in addition to the usual conditions of temperature, incubation, time, etc.

The k_2 value (and hence the various possible I_{50}'s) for an inhibitor against a particular cholinesterase will vary with pH and temperature. The effect of pH upon TEPP inhibition has been utilized by Wilson to explore the nature of the active surface of electric eel true cholinesterase (Fig. 3.4). He argues that as the structure of TEPP is unaltered by pH, the variation shown in Fig. 3.4 reflects modifications in the ionized nature of the active centers. This has been discussed above (page 17). For the present, it is sufficient to note that there is a pH optimum for organophosphorus inhibition purely as a result of changes in the esteratic site. If the organophosphate contains a group which contributes to the affinity for cholinesterase by combining with the anionic site of the enzyme, the picture will be more complex, because the charge situation of that site will also vary with pH.[17] If the anionic-complexing group on the phosphate is, say, a quaternary nitrogen (e.g., phosphopyristigmine), it will not itself alter

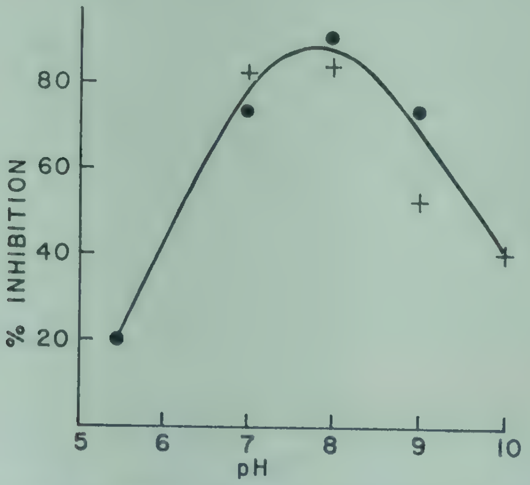


FIG. 3.4. Relationship between pH and inhibition of acetylcholinesterase by TEPP. From Wilson and Bergmann.[100]

with pH. But if it is a tertiary nitrogen (e.g., Tetram), it too will vary in structure—or protonation—with pH; this will modify its affinity for the site, and the variability will be superimposed upon the other pH-variant effects.[81]

The reaction between organophosphates and cholinesterase is a chemical reaction, and therefore its rate will increase with temperature. At temperatures in excess of the optimum for enzyme activity, the picture will be complex because of heat-denaturation of the enzyme. The variation with temperatures of 17.6–36.7°C. of the inhibition of rabbit erythrocyte cholinesterase by paroxon has been studied briefly by Aldridge,[5] and from these data an energy of activation of 10.6 kcal. mole^{-1} was observed. Other energies of activation have been calculated, e.g., 14.5 kcal. mole^{-1} for N,N'-diisopropyl phosphorodiamidic anhydride against rat jejunum pseudocholinesterase.[34]

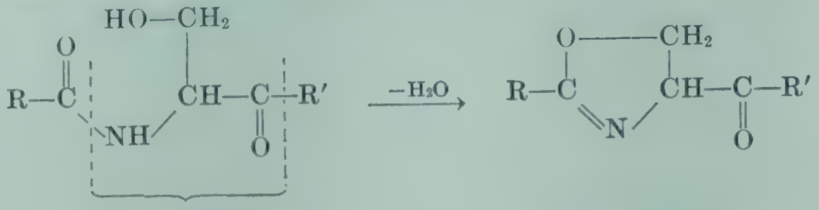
(*b*) *Site.* It is clear that organophosphate inhibition of cholinesterase involves a phosphorylation. What substituent in the enzyme molecule is phosphorylated? A number of pieces of evidence from different sources suggest that the primary phosphorylation is of histidine:

(1) Of all the common amino acids, only histidine and tyrosine react with DFP under neutral aqueous conditions. The reaction with histidine[94] gives an unstable intermediate, so that the observed effect is that histidine catalyzes DFP hydrolysis. The reaction with tyrosine[65] yields a stable *O*-phosphoryl derivative. But degradation, enzymic or acid, of DFP^{32}-treated esterases shows all the P^{32} bound to serine.[29, 87, 88] If tyrosine were the primarily attacked acid, one would expect to find the stable tyrosine phosphate in hydrolyzates; but if histidine were attacked, it might easily transphosphorylate, subsequently, to give serine phosphate.[94]

(2) Evidence, so far, suggests that the esteratic sites of chymotrypsin and cholinesterase are very similar.[16] Slow photo-oxidation of chymotrypsin in the presence of methylene blue led to loss of only histidine and tryptophan along with a loss in enzyme activity. The inactivated enzyme could no longer react with DFP.[64, 95] Peroxidase treatment of chymotrypsin led to the loss of tryptophan only; enzymic activity was only partially lost and the treated enzyme reacted with diphenyl phosphorochloridate just as easily as did the untreated.[105]

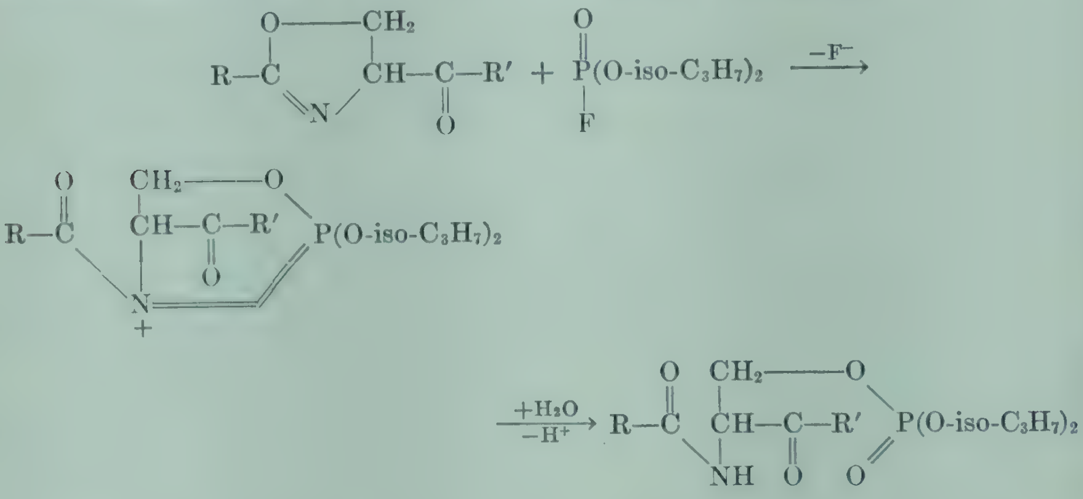
(3) Examination of the way in which pH effects hydrolysis by cholinesterase suggests that there is a basic group in the esteratic site of pK_a about 6.5[16, 75] and only the pK_a imidazole ring of histidine falls in this range.

A contrary view has been put forward by Porter *et al.*[83a] They suggest that the serine residue in the active center may be present as an anhydride with the carbonyl group of its neighboring amino acid, giving a Δ^2-oxazoline:



Serine Δ^2-Oxazoline

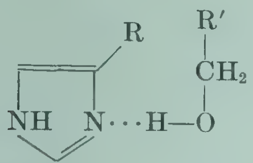
They made three Δ^2-oxazolines, and found that if the nitrogen was basic enough (e.g., pK_a of 5) they would react with DFP in an aqueous alkaline medium (under which conditions serine is unreactive):



They feel, therefore, that the serine of the cholinesterase esteratic site may be in this form and may be directly attacked by organophosphates. The argument is weakened by the requirement for alkaline conditions for reaction, and should be regarded (as they put it forward) as a tentative working hypothesis.

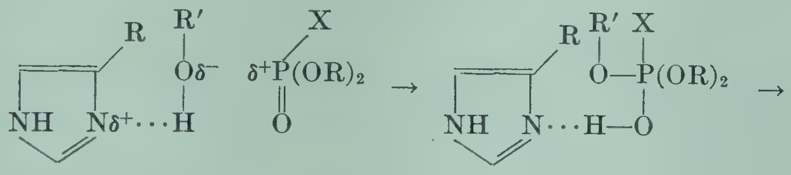
A third proposal comes from Cunningham,[31] who considered that although the pK of the phosphorylatable group in the active center of chymotrypsin favored the idea that histidine was involved, the heat of ionization of the group (found by studying the variation of pK with temperature) was unduly high for histidine. Like Porter *et al.*, Cunningham suggested that a "modified serine" was in fact involved; but his suggestion was that the serine was "modified" by an adjacent imidazole ring (probably of histidine). His proposal should apply also to cholinesterase.

Cunningham suggested that the oxygen of the serine hydroxyl might be modified by hydrogen bonding with a hydrogen from a nearby imidazole ring.

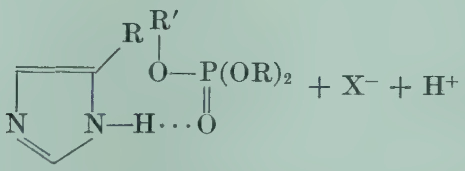


Histidine Serine

The consequent reduction of the influence of the positive hydrogen should make the oxygen somewhat more negative, and, thus, increase its sensitivity to attack by the positive phosphorus atom of the organophosphate. The subsequent reactions would be:



Enzyme active center



Serine-phosphorylated enzyme

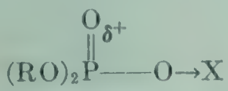
Some further comments upon the nature of the phosphorylated amino acids will be found at the end of the section on "aging."

c. *The Relationship between Structure and Anticholinesterase Activity*

There are two important factors deciding the *in vitro* anticholinesterase properties of an organophosphate. First, there is the affinity of the compound for the active site; secondly, there is the readiness with which the compound, once arrived at the site, can phosphorylate it. In long-term experiments one would also have to consider the ease of subsequent regeneration of the enzyme, but this topic will be given a section of its own below.

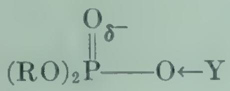
Let us consider first the ability to phosphorylate the site. This phosphorylation is a straightforward chemical reaction, as the last section showed. It turns out that the organophosphate is acting as an electrophilic reagent (it is making an electrophilic attack): that is to say, the phosphorus atom is attaching itself to an atom of relatively high electron density. This is precisely what was going on in the hydrolytic reaction discussed in Chapter 2: the reaction consisted initially of an attack of OH^- upon P. It can be looked up on alternatively as an attack of the P upon the OH^-. (When a nucleophile is attacking, the attack is said to be nucleophilic; when an electrophile is attacking the attack is said to be electrophilic. Which species we choose to call the attacker is of course arbitrary. One tends, other things being equal, to call the smaller molecule the attacker. For this reason, we commonly talk of the nucleophilic attack of OH^- upon a phosphate, but of the electrophilic attack of a phosphate upon cholinesterase; yet these processes are really precisely analogous.) The main basis for saying that the inhibition of cholinesterase is an electrophilic attack is the observation that within certain limits the substituents upon the phosphorus which increase the alkaline hydrolytic rate are precisely those that increase the rate of inhibition of cholinesterase.

It would be a reasonable guess that those substituents which when attached to the P make it a better electrophilic reagent (i.e., more positive), will improve its anticholinesterase activity.



$$(\mathrm{RO})_2\overset{\overset{\mathrm{O}\ \delta+}{\|}}{\mathrm{P}}\text{——}\mathrm{O}\rightarrow\mathrm{X}$$

Improved anticholinesterase



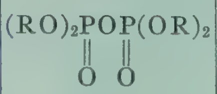
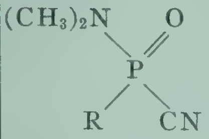
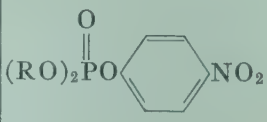
$$(\mathrm{RO})_2\overset{\overset{\mathrm{O}\ \delta-}{\|}}{\mathrm{P}}\text{——}\mathrm{O}\leftarrow\mathrm{Y}$$

Worsened anticholinesterase

Therefore, in general, it might be expected that the more electrophilic the substituents, the better the anticholinesterase activity. We should, therefore, expect that in $(RO)_2P(O)OX$ (*a*) the anticholinesterase activity would fall off in the order R = methyl > ethyl > propyl > butyl, etc.; (*b*) replacing RO by R should reduce activity; (*c*) the activity would increase with the electrophilic character of X. Looking at it another way, this is the same as saying that it should increase with the acid character of the compound XOH. Thus, since *p*-nitrophenol is a stronger acid than phenol, then dialkyl *p*-nitrophenyl phosphates should be better anticholinesterases than dialkyl phenyl phosphates. The common factor is of course that electrophilic substituents in X make XOH a stronger acid (compare acetic with chloracetic acid) and make $(RO)_2POX$ a better anticholinesterase; (*d*) finally, we may expect that $(RO)_2P(S)OX$ will be a weaker anticholinesterase than $(RO)_2P(O)OX$, because $=S$ is a weaker electrophile than $=O$ (or, to be a little more precise, the difference in electronegativity between P and S is less than that between P and O). Let us examine these factors in order.

(*a*) Table 3.1 shows the effect of changes in the alkyl substituents. A large pI_{50} means an efficient inhibitor. It is very certain that the above prediction does not hold. In two of the classes shown the maximum anticholinesterase activity is given by the isopropyl compounds, in the other class by the ethyl compounds. The only explanation that can be tentatively offered to explain the failure of prediction is that dimethyl phosphorylated cholinesterase is fairly readily reactivated after inhibition, and that di-

TABLE 3.1. EFFECT OF ALKYL GROUPS UPON ANTICHOLINESTERASE ACTIVITY (pI_{50})[a]

R	$(RO)_2POF$	 $(RO)_2P(O)OP(O)(OR)_2$		
CH_3	7.0	5.7	7.8	6.8
C_2H_5	8.1	9.1	8.4	7.3
n-C_3H_7	8.25	7.6	—	7.6
iso-C_3H_7	8.9	—	8.9	6.0
n-C_4H_9	—	8.9	—	7.8
sec-C_4H_9	8.7	—	—	6.3
iso-C_5H_{11}	8.7	—	—	—
Enzyme	Horse serum	Human serum	Human erythrocytes	"True cholinesterase"
Incubation time (minutes)	15	Not stated	ca. 25	30
Incubation temperature (°C)	20	25	25	25

[a] Data of Mackworth and Webb,[70] Brauer,[21] Holmstedt,[61] and Ooms *et al.*[82]
pI_{50} = negative logarithm of molar concentration for 50% enzyme inhibition.

isopropyl phosphorylated enzyme recovers not at all (see section below on reversal). There is no direct proof, however, that this is the cause of the failure.

(*b*) There is no data to permit a full comparison of $(RO)_2POX$ with either $(RO)RPOX$ or R_2POX as anticholinesterases. Fukuto and Metcalf[41] report pI_{50}'s of 7.6 and 7.9 respectively for diethyl *p*-nitrophenyl phosphate and ethyl *p*-nitrophenyl ethylphosphonate. In this case, again, this difference (if significant) goes in a contrary direction to prediction. No information is available of the recovery rate of phosphorylated cholinesterase, so that the failure of prediction cannot be explained.

(*c*) It is a relief to turn to a study of variations in the group X of, e.g., $(RO)_2POX$, for here prediction is (within limits) a great deal more reliable. The reason is, probably, that one naturally compares different X's while holding R constant, and the recovery factor is identical within such a series of compounds (since all give the same alkyl phosphorylated enzyme). Four criteria have been used in the literature to measure the electrophilic nature of X: the acid strength of ROX; the alkaline hydrolyzability of $(RO)_2 \cdot P(O)OX$; the sigma constant of substituents when X is a substituted phenyl group; and the infrared absorption of P—O—X.

Ketelaar[67] used the acid strength concept in 1951. Table 3.2 shows examples in the substituted aromatic diethyl phosphates: when the derived

TABLE 3.2. RELATIONSHIP BETWEEN ANTICHOLINESTERASE ACTIVITY OF $(RO)_2P(O)OX$ AND ACID STRENGTH OF XOH[a, b]

Y=	pK of HO	pI_{50} of $(C_2H_5O)_2P(O)O$
p-NH_2	10.3	4.0
—	9.9	3.5
p-Cl	9.2	3.4
o-Cl	8.5	4.2
o-NO_2	7.2	6.5
p-NO_2	7.2	7.7

[a] High pK = weak acid. High pI_{50} = good anticholinesterase.
[b] From Ketelaar[67] and Ahmed *et al.*[2]

phenol XOH is a strong acid, then the phosphate tends to be a good inhibitor. As the table shows, the correlation is only qualitative. The pK of the acid XOH, is, of course, a measure of the electrophilic nature of X: when X is strongly electrophilic, it draws electrons strongly from the oxygen and increases the tendency of the hydrogen to ionize, and XOH is then a "strong" acid.

The second way of expressing the electrophilic nature of X is by examining the alkaline hydrolyzability of $(RO)_2P(O)OX$. Chapter 2 showed that in general the more electrophilic was X, the more sensitive to OH^- was the organophosphate. The first quantitative expression of this concept was by Aldridge and Davison[7] who demonstrated that in a series of ten diethyl

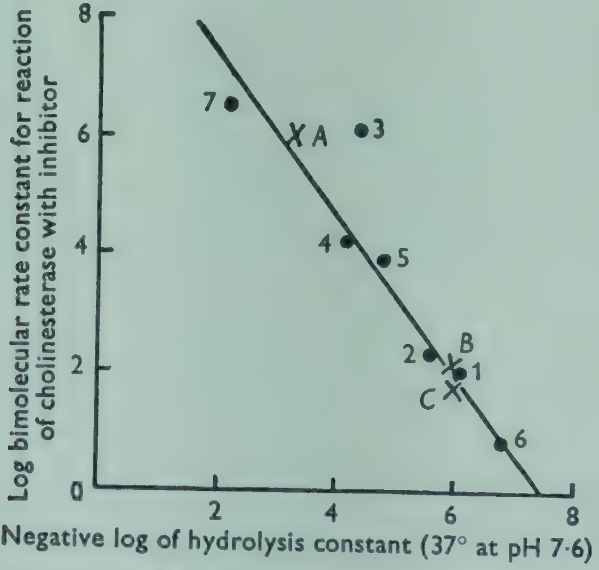
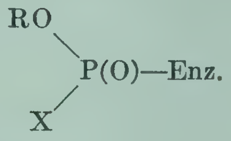


FIG. 3.5. Relationship between hydrolyzability and anticholinesterase activity for ten substituted diethyl phenyl phosphates. From Aldridge and Davison.[8]

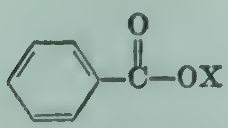
substituted phenyl phosphates there was a remarkably linear correlation between the logarithms of the bimolecular rate constant for cholinesterase inhibition and the logarithms of the hydrolysis constant (Fig. 3.5). The more stable to alkaline hydrolysis were the phosphates, the less effective were they as inhibitors of cholinesterase. The data of Fukuto and Metcalf[40] with some other phenyl phosphates reveals a similar correlation. Clearly, in both inhibition and hydrolysis the substituent effects are similar: electrophilic groups such as Cl and NO_2 draw electrons away from the phosphorus and make it more positive and, therefore, more open to attack by OH^- and more able to attack the electron-dense area in cholinesterase.

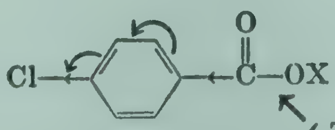
Fukuto and Metcalf[41] also studied this effect in quite a different series of compounds: phosphonates (RO)(R′O)P(O)X where R was ethyl, R′ was *p*-nitrophenyl, and X was varied. Seventeen compounds were made, and a moderately good correlation between anticholinesterase activity and hydrolyzability was found. That this correlation is only moderate may be due to the fact that in this case the phosphorylated enzyme may well be



and thus in this series the inhibited enzyme is not the same for every member.

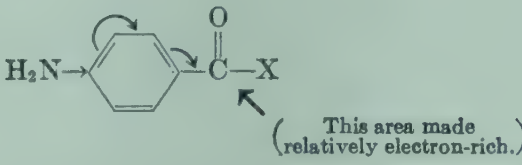
The electron-withdrawing capacity of substituents in aromatic compounds has been quantitatively evaluated by Hammett,[53] who has allocated a sigma constant (substituent constant, σ) to the common groups. These constants were calculated from certain equilibria and reaction rates of substituted phenyl compounds, and can be used with considerable accuracy to predict how the substituents will affect other equilibria and rates. For example, chlorine is electrophilic, and therefore its presence in a phenyl compound will enhance reactions in which the compound's active center should be electrophilic (for the chlorine draws electrons from the center, and the center thereby becomes more avid for electrons). Clearly the chlorine will proportionately depress those reactions (or equilibria) in which the compound's active center should be nucleophilic. For instance, substitution by chlorine (whose sigma constant is positive) would increase the susceptibility of an ester to hydrolysis catalyzed by OH^-, but decrease its susceptibility to hydrolysis catalyzed by H^+:





This area made relatively electron-deficient.

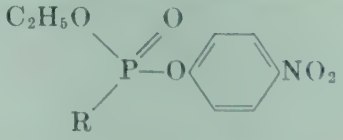
On the contrary, NH_2 (whose sigma constant is negative) would enhance the susceptibility to H^+ hydrolysis, but decrease its susceptibility to OH^- hydrolysis.



Why should only aromatic compounds be used? The reason is that only then do steric effects become (in most cases) negligible. Even in aromatic compounds the so-called "ortho" effect occurs: substituents in the ortho-position frequently give atypical results because of steric interference. In aliphatic compounds one does not have the rigid skeleton that keeps the substituents away from the reaction center of the molecule, and quantitation becomes less reliable. As Chapter 2 shows, however, qualitative predictions can be very effective.

Sigma constants were first used in describing the anticholinesterase action of organophosphates by Fukuto and Metcalf in 1956.[40] With two exceptions, there was a good correlation between anticholinesterase activity and the sigma constant of the substituents in a series of 17 aromatic diethyl phosphates, as shown in Fig. 3.6. The exceptions were readily explained by steric factors (discussed below). The Riverside group[15] later applied the same method successfully to derivatives of Bayer 25141. The introduction of the sigma constant to this field was a fine contribution. It expresses directly what the other methods (acid strength, hydrolyzability) reflect indirectly. It is unfortunate that it can only be used with aromatic compounds.

Fukuto *et al.*[45] have more recently applied a similar approach to variations in the R group of various ethyl *p*-nitrophenyl alkylphosphonates:



The Hammett sigma constant σ cannot apply to such cases, since R is not an aromatic substituent. The Taft sigma constant σ^* (or "polar substituent constant") was used instead; this is a parameter essentially equivalent to the Hammett sigma constant, but applicable to aliphatic and *ortho*-aromatic substituents. Fukuto *et al.*[44] found a rough correlation between σ^* of the various R's and the toxicity of the various compounds to houseflies, which in turn they had shown[41] to be correlated with hydrolepability which in turn was correlated with anticholinesterase activity. A direct plot of σ^* against anticholinesterase shows, however, absolutely no correlation: for instance although the σ^* values for n-C_4H_9, iso-C_4H_9, n-C_3H_7 and C_2H_5 vary 1.3 fold (range −0.100 to −0.130) the anticholinesterase activities of the corresponding phosphonate vary 26-fold. Even the correlation between σ^* and toxicity is slight, if from the published figure (ref.[44]) one removes the point for R = $C(CH_3)_3$ for which no value was established[41, 45] and adds points for R = $CHCl_2$ and C_6H_5 which were tabulated but not figured.

Finally, there is the infrared method, which has only been used by

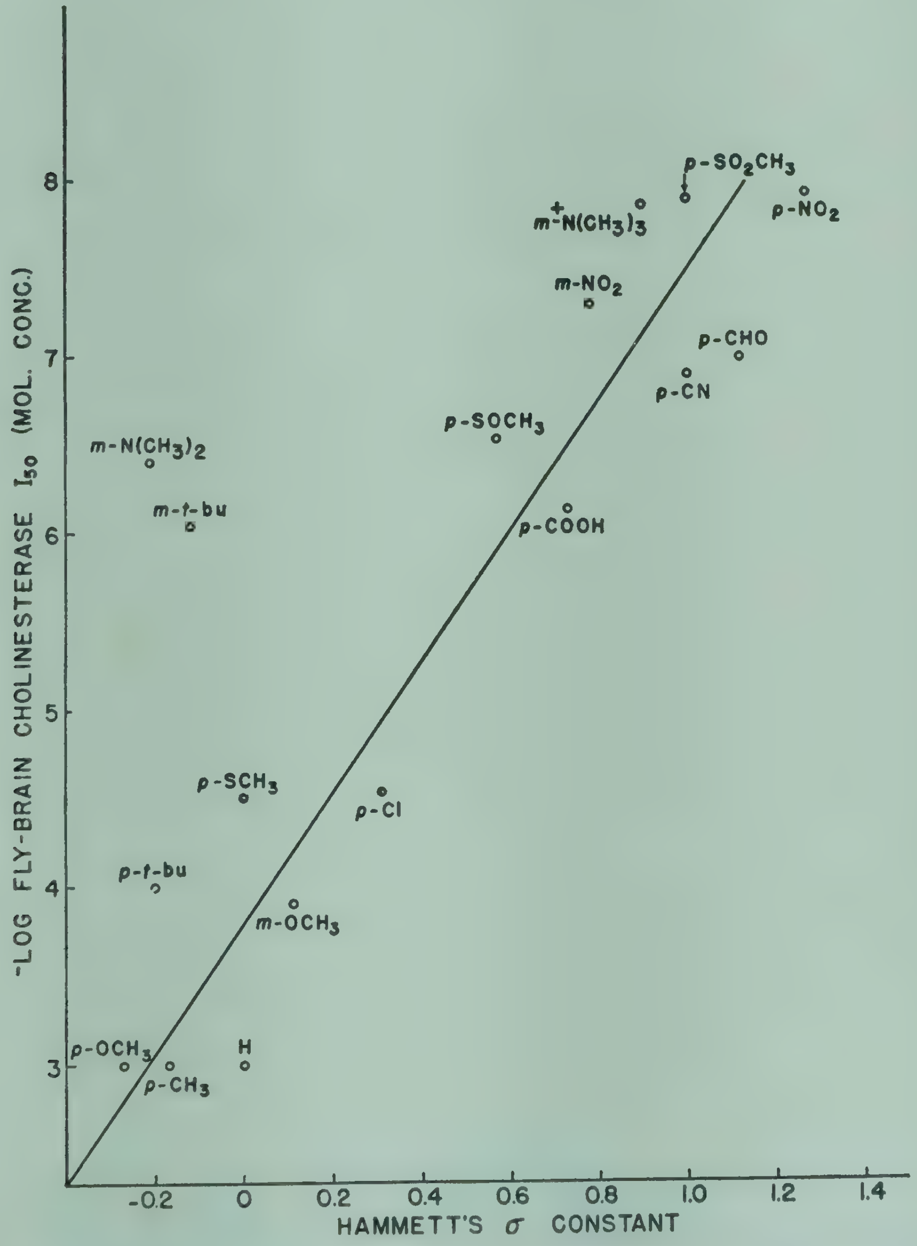


FIG. 3.6. Relationship between anticholinesterase activity and Hammett's σ constants for substituted diethyl phenyl phosphates. From Fukuto and Metcalf.[40]

Fukuto and Metcalf.[40] They found a good correlation in their diethyl phenyl phosphates between anticholinesterase activity and the frequency of the P—O aromatic bond, as shown in Fig. 3.7. However, there is one puzzling feature. One would expect that when X in $(RO)_2P(O)OX$ was very electrophilic, it would draw electrons away from the P—O—X bonds, weakening them and reducing the frequency of the P—O—X stretching frequency (these bonds may be considered rather like springs: when a spring is weak, its frequency of oscillation is small). This was indeed found to be the case

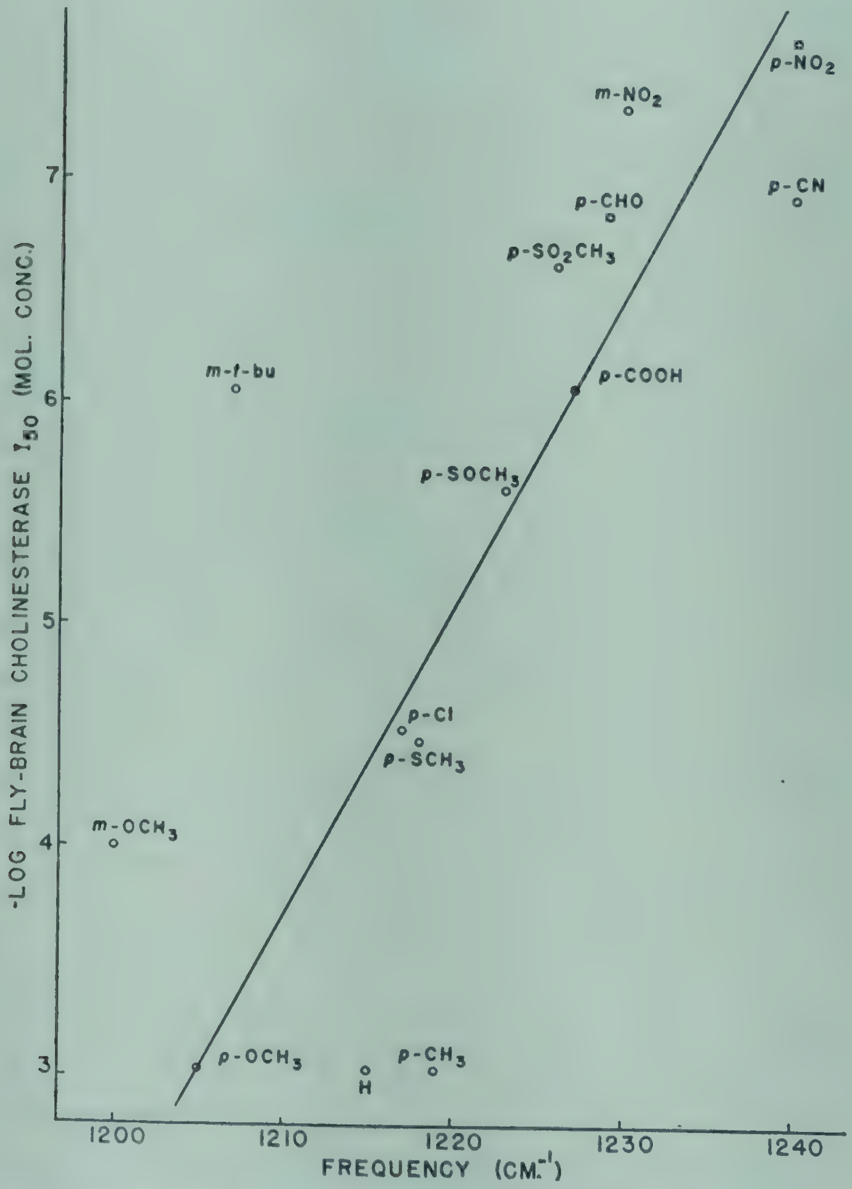


FIG. 3.7. Relationship between anticholinesterase activity and infrared P-O-aromatic stretching frequency for substituted diethyl phenyl phosphates. From Fukuto and Metcalf.[40]

TABLE 3.3. EFFECT OF CONVERSION OF P=S TO P=O UPON ANTICHOLINESTERASE ACTIVITY

P=S form			P=O Isomer		P=O oxidation product	Cholinesterase	Reference
$(RO)_2P(S)OR'$			Isomer form	pI_{50}	pI_{50}		
R=	R'=	pI_{50}					
CH_3	$C_6H_4NO_2(p)$	2	$(RO)_2P(O)SR'$	6.5	6.0	Serum	12
C_2H_5	$C_6H_4NO_2(p)$	4	$(RO)(RS)P(O)OR'$ $(RO)_2P(O)SR'$	6 8.55	8.18	Serum	12
C_2H_5	$(CH_2)_2N(CH_3)_2$	4.4	$(RO)_2P(O)SR$	8.12	5.5		91
C_2H_5	$(CH_2)_2S(C_2H_5)$	3.7	$(RO)_2P(O)SR'$	5.5	7.6	Fly brain	43
C_2H_5	$(CH_2)_2S(O)C_2H_5$	5.5	$(RO)_2P(O)SR'$	5.8	6.0	Fly brain	43
C_2H_5	$(CH_2)_2S(O)(O)C_2H_5$	6.1	$(RO)_2P(O)SR'$	6.2	6.9	Fly brain	43
CH_3	$CHCOOC_2H_5$ | $CH_2COOC_2H_5$	2.01	$(RO)(RS)P(O)SR'$	4.5	6.35	Serum	78, 80

for the H—O-aromatic stretching frequency of substituted phenolic compounds (Ingraham *et al.*[62]) but is the opposite of the case found by Fukuto and Metcalf. At present there is no explanation for the anomaly.

(*d*) A most important modification of organophosphorus molecules is the conversion of P=S to P=O. This change, which may be accomplished either by isomerization [e.g., of $(RO)_2P(S)OX$ to $(RO)_2P(O)SX$], by chemical, or by enzymic oxidation, always makes the compound less stable in water and a better anticholinesterase. Several examples are given in Table 3.3 (the effect of the conversion upon hydrolyzability is discussed in Chapter 2). It will be noted that if the P=S compound is already a potent inhibitor, the oxidation or isomerization product is only a little better than the parent as an anticholinesterase.

Another oxidation that is of importance is the enzymic or chemical oxidation of a mercapto sulfur in Systox-type compounds, either to a sulfoxide [—S(O)R] or a sulfone [—S(O)(O)R]. The anticholinesterase activity of the mercapto, sulfoxide, and sulfone derivatives of Systox thiono isomer, thiolo isomer, and oxidized thiono isomer, will be found in Table 3.3 (fourth, fifth, and sixth compounds, reading vertically). Here it can be seen that these progressive oxidations lead to progressive increases in anticholinesterase activity for the thiono and (to a lesser extent) for the thiolo isomer; but that mercapto oxidation of the already potent oxidized thiono isomer leads to a decrease in ability.

This leads on to a less happy part of this story: the account of the certain failures of correlation between hydrolyzability and anticholinesterase activity. Burgen[23] had pointed out, earlier, that in the series, dimethyl, diethyl, and diisopropyl phosphorofluoridates, the hydrolyzability decreased in that order, yet the anticholinesterase activity increased. Also DFP is a worse phosphorylator yet a better anticholinesterase than its chlorine analog. To explain these paradoxical observations, Burgen suggested that in the less stable agents "the rate of reaction with the enzyme will be increased, but the rate of nonspecific action such as phosphorylation of random amino hydroxyl or phenolic groups may increase, as will hydrolysis by water molecules. These side reactions will divert some of the active agent—an important matter with substances affective at very low concentrations."

It follows that if one takes a weak anticholinesterase and progressively substitutes electrophilic groups into it, the hydrolyzability will steadily increase; but the anticholinesterase activity will increase, pass through an optimum, and decrease again. Such behavior has been shown by the progressive chlorination of schradan;[89] the first chlorine introduced enormously increased hydrolyzability and anticholinesterase activity; but further chlorination made the compound excessively hydrolyzable and a poor inhibitor (Fig. 3.8).

Such an excessive instability may account for the failure, mentioned above, of mercapto oxidation to increase the inhibitory activity of oxidized thiono-Systox. Certainly it would be important for the tertiary analogs of the cholinyl methylphosphonofluoridates of Tammelin[90] which are excessively unstable. Tammelin also reports that the fact that the quaternary analogs are not better anticholinesterases than the related compound sarin is attributable to their short half-life in water—9 minutes and 23 minutes for two of them, compared with 320 minutes for sarin.

At the other extreme, there is such a compound as the phosphonate Armin [$Et(EtO)P(O)OC_6H_4pNO_2$] which is claimed by Razumov[85] to be stable in aqueous solution for years and "shows anticholinesterase inhibition at 1×10^{-7} to 2×10^{-9} M." At the moment we cannot explain such properties.

Finally in this discussion a fact must be mentioned which is a commonplace experience, yet is a keystone for the invention of selectively toxic compounds, as Chapter 9 will show. Because nucleophilic groups inserted on X depress anticholinesterase activity, and because an anion is an ex-

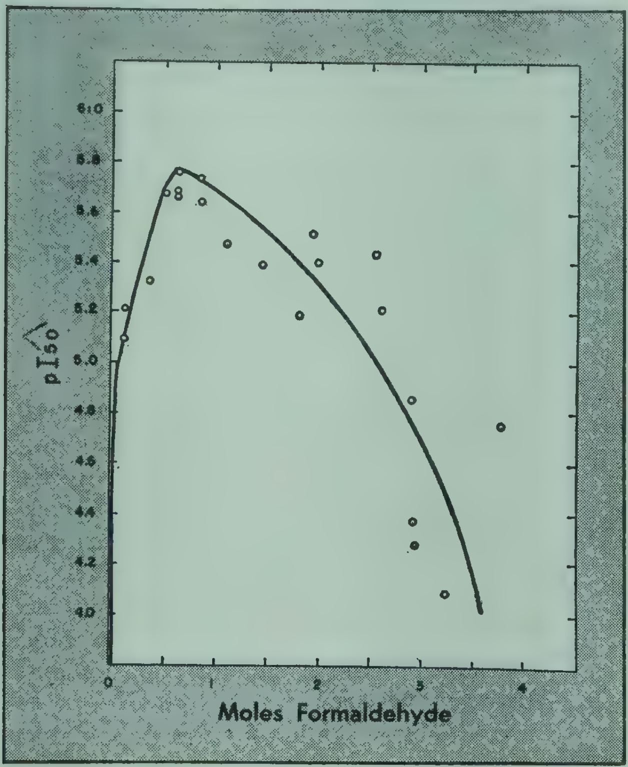


FIG. 3.8. Relationship between anticholinesterase activity and average degree of chlorination per mole of schradan, as measured by subsequent formaldehyde release. From Spencer and O'Brien.[89]

tremely strong nucleophile, any hydrolysis which leaves an anionic group near the phosphorus will eliminate anticholinesterase activity. For this reason the following hydrolyses will eliminate activity:

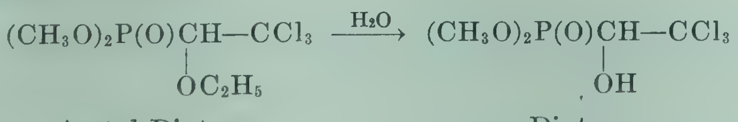
$$(C_2H_5O)_2P(O)OP(O)(OC_2H_5)_2 \xrightarrow{H_2O} 2(C_2H_5O)_2P(O)O^-$$

TEPP

$$(C_2H_5O)_2P(O)SCH_2COOC_2H_5 \xrightarrow{H_2O} (C_2H_5O)_2P(O)SCH_2COO^-$$

Acetoxon

These products will, of course, be ionized under physiological conditions. Certain hydrolyses of some molecules do not produce anions, and therefore do not eliminate toxicity:[9a]

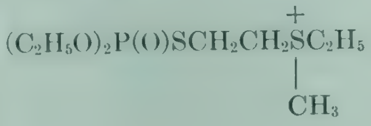
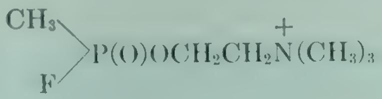
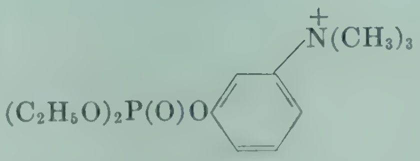
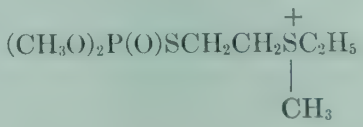


$$(CH_3O)_2P(O)\underset{\displaystyle OC_2H_5}{\underset{|}{C}}H{-}CCl_3 \xrightarrow{H_2O} (CH_3O)_2P(O)\underset{\displaystyle OH}{\underset{|}{C}}H{-}CCl_3$$

Acetyl Dipterex Dipterex

So far, we have only discussed the factors governing the phosphorylating ability of organophosphates. If, however, for any reason, electrostatic or steric, some other part of the organophosphate should have an impor-

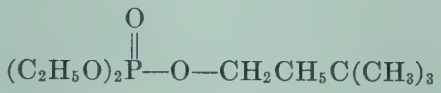
TABLE 3.4. ORGANOPHOSPHATES WITH ACETYLCHOLINE-LIKE STRUCTURE

Compound	Structure	pI_{50}	Cholinesterase	Reference
Acetylcholine	$CH_3C(O)OCH_2CH_2\overset{+}{N}(CH_3)_3$			
Tetram (protonated form)	$(C_2H_5O)_2P(O)SCH_2CH_2\overset{H+}{N}(C_2H_5)_2$	10.3	Plasma	81
Quaternarized Tetram	$(C_2H_5O)_2P(O)SCH_2CH_2\overset{+}{N}(C_2H_5)_3$	9.3	Plasma	81
Thiolo-Systox methosulfonium derivative	 $(C_2H_5O)_2P(O)SCH_2CH_2\underset{\underset{CH_3}{\mid}}{\overset{+}{S}}C_2H_5$	7.5	Housefly head	44
Cholinyl methylphosphonofluoridate		10.0	Plasma	90
Diethyl phosphostigmine		8.9	Plasma	24
Thiolo methyl Systox methosulfonium derivative	 $(CH_3O)_2P(O)SCH_2CH_2\underset{\underset{CH_3}{\mid}}{\overset{+}{S}}C_2H_5$	7.4	Red cells	55

tant influence upon the approach of the molecule to the enzyme, we must expect departures from simple prediction. From the description of cholinesterase in Chapter 1, the following guesses could be made: (*1*) a conveniently placed cationic group on the phosphates might bind to the anionic site of the enzyme, and thereby increase the effectiveness of the phosphate; (*2*) any bulky groups near the P might interfere with the approach of the phosphate to the enzyme and decrease its effectiveness.

What is a "conveniently placed cationic group?" Since the optimal substrate for cholinesterase is acetylcholine, it is probable that the separation of the cation by the distance of an —O—C—C— chain (as in acetylcholine) from the group binding to the esteratic site, i.e., the P=O group, would be optimal. Table 3.4 lists compounds which fit this specification, and indeed they are all extraordinarily potent anticholinesterases.

Fukuto and Metcalf,[40] in their study on the relationship between electrophilic character and anticholinesterase activity of various substituted phenyl phosphates, noted that of the seventeen studied, two had I_{50} values about 1000-fold less (i.e., were 1000 times more potent) than expected. These were the *m*-dimethylamino and the *m*-tertiary butyl derivatives. These two groups, therefore, probably assist in the orientation of inhibitor on enzyme, presumably by complexing with the anionic site. A confirmation is the observation[42] that the compound



$$(C_2H_5O)_2\overset{\overset{\displaystyle O}{\|}}{P}{-}O{-}CH_2CH_5C(CH_3)_3$$

is an unexpectedly potent anticholinesterase, with an I_{50} of 3×10^{-7} (unexpected because the tertiary hexyl chain is nucleophilic). The fact that a tertiary butyl group can bind to the anionic site is rather surprising. It is true that

$$-C(CH_3)_3$$

is sterically like

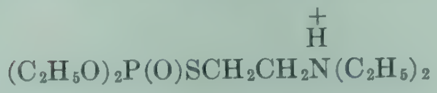
$$-\overset{+}{N}(CH_3)_3$$

but the electronic properties of the C are totally unlike those of the N^+. It may be that there are two separable effects involved in binding to the anionic site: (1) steric, important because of short-range Van der Waals' binding of precisely positioned carbons; (2) electrostatic. The existence of a "pure" electrostatic effect cannot be demonstrated at present; although appropriately placed positive changes have been introduced on N and S (Table 3.4) quaternarization has involved introduction of an additional alkyl group as well as a charge, in comparison with their uncharged analog. Thus, both steric and electrostatic improvements have been made. In the case of substrate binding, the case has, however, been studied: Wilson[98] finds vigorous hydrolysis of dimethylaminoethyl acetate only at such pH's that the substance is present in the protonated (and therefore charged) form. The steric difference between the protonated and unprotonated form is, of course, small.

One would predict, then, that the tertiary nitrogenous inhibitor Tetram[46] would inhibit well at low pH values (protonated form) and progressively less as the pH increases, until the free base is the preponderant species. This has been confirmed:[82]

$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$

Tetram at high pH (poor inhibitor)



$(C_2H_5O)_2P(O)SCH_2CH_2\overset{\overset{+}{H}}{N}(C_2H_5)_2$

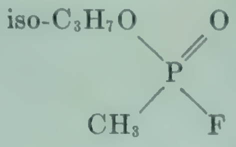
Tetram at low pH (good inhibitor)

The steric factors have not been studied in great detail for organophosphorus inhibitors, but there is a good deal of information from specificity studies upon a variety of substrates and inhibitors designed specifically to explore the nature of the active sites. This is not a treatise on cholinesterase, so the details of these studies cannot be discussed; but some of the conclusions emerging from them should play an important part in the design of organophosphate inhibitors. The series of papers of Friess and his associates (e.g., refs.[37-39]) make it clear that it is by no means essential to have a quaternary group to combine with the anionic and a carbonyl to react with the esteratic site. What is required for complexing with cholinesterase is "a locus of high electron density separated by roughly a —CH_2CH_2–unit's distance from a polymethylated nitrogen atom (preferably quaternary.)"[38] Undoubtedly, however, cationic groups other than a quaternary nitrogen will complex with the anionic site (e.g., in iso-Systox methosulfate).

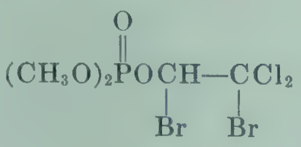
Since true cholinesterase has more than one anionic site (page 16) it should be possible to make organophosphates containing two appropriately situated quaternary groups, and greatly increase the anticholinesterase activity as a result of multiple binding.

A little work has been done upon the specificity of optical isomers of organophosphates. Michel[73] in 1955 reported that pure sarin inhibited cholinesterase as if two components were present in equal amounts, one of which inhibited slowly, the other rapidly. In 1958, Aaron *et al.*[1] obtained the two optical isomers of the phosphonate $(EtO)(Et)P(O)S\ CH_2CH_2SEt$, and found that the *l* isomer was from 10 to 20 times the better anticholinesterase, depending on the enzyme source (Table 3.5).

Optical isomerism occurs whenever a tetravalent atom has four different substituents. One may therefore expect a "two component" effect arising from phosphorus isomerism in many phosphonates, such as EPN, Sarin, and Soman, and from carbon isomerism in such compounds as butonate, Dibrom, Dipterex and malathion.



Sarin



$(CH_3O)_2\overset{O}{\overset{\|}{P}}O\underset{Br}{CH}-\underset{Br}{CCl_2}$

Dibrom

TABLE 3.5. ANTICHOLINESTERASE ACTION OF OPTICAL ISOMERS OF *O*-ETHYL *S*-(β-ETHYLMERCAPTOETHYL) ETHYLPHOSPHONOTHIOLATE

Cholinesterase source	Velocity constant of inhibitory reaction ($k \times 10^{-2}$ M sec.$^{-1}$)			
	Temperature (°C)	*l*-form	*d*-form	ratio
Electric eel	25	14.3	0.78	18
Electric eel	15.1	7.3	0.37	20
Human red cells	25	13.2	1.37	9.6
Bovine red cells	25	9.0	0.94	9.6
Horse serum	25	5.0	0.25	20

[a] Data of Aaron *et al.*[1]

d. Protection from Inhibition

It has been known for a long time that acetylcholine can protect cholinesterase from inhibition by organophosphates, and this has been universally confirmed. About the extent of the protection, there is some disagreement. Burgen[23] reported that with TEPP, inhibition was completely prevented by prior or simultaneous addition of either acetylcholine or acetyl-β-methylcholine, even if TEPP was at 100 times the level required to produce 50% inhibition in an unprotected system. However, with DFP, the protection was not complete, and although protection was found, when DFP was at this "hundredfold" level (in this case, about 3×10^{-5} M) there was considerable inhibition even in the presence of 0.02 M acetylcholine. Similar incomplete protection was reported by Hobbiger for acetyl-β-methylcholine against 3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methosulfate.[57] Although the substrate (0.03 M) protected completely against 5×10^{-10} M inhibitor, it was withought effect on 5×10^{-8} M inhibitor. Augustinsson and Nachmansohn[13] found an almost complete protection against 10^{-8} M TEPP by 3×10^{-3} M acetylcholine when these were added together, but with a series of DFP concentrations up to 10^{-3} M, the protection was less and less complete.

Roan and Maeda,[86] working with eight organophosphates and fruit-fly cholinesterase, observed that protection was twice as effective if acetylcholine was added before than if it was added together with the inhibitor, and that even with prior acetylcholine addition, protection was only complete in the lower range of inhibitor concentrations.

Other work has, however, suggested that complete protection is normally found. Mackworth and Webb[70] working with pseudocholinesterase and DFP, and using a number of acetylcholine concentrations from 3×10^{-3} up to 0.1 M, reported that "the fluorophosphonate gives no indication of competition with the substrate"—which was added after the inhibitor (in the case of eserine, considerable competition with substrate was of course observed). Aldridge[3] found that paraoxon and DFP at about 10^{-6} M, normally inhibiting true cholinesterase by 60 and 70% respectively, only inhibited by 3% if added after acetylcholine (10^{-2} M). The subsequent kinetic studies, based on this assumption, of this worker and his collaborator Davison, have given excellent results, and one cannot doubt that it is a valid assumption for their conditions. However, all these studies have, of course, been made with inhibitor levels and incubation times designed to give less than 100% inhibition of the unprotected enzyme. There is, therefore, no reason to doubt that with organophosphate levels higher than that required to produce 100% inhibition of the unprotected enzyme, prior addition of substrate will not always protect completely; and this incomplete protection will probably be particularly noted with DFP, and less with TEPP.

The simplest empirical test to ensure that inhibition has ceased upon addition of substrate is to see if the plot of cholinesterase activity against time (e.g., μl. of CO_2 per minute, in the Warburg assay) is linear.

An ingenious use of the high specificity and protective action of benzoylcholine has been made by Cohen and Warringa.[30] These workers wanted to bind labeled DFP to the active sites of cholinesterase, and, thus, find the number of sites in their enzymic preparation; with this information and a knowledge of the activity of their enzyme they could calculate the turnover number. However, when organophosphates are incubated with cholinesterase, a good deal of the phosphorylation that occurs is of amino acids not in the active site. They therefore incubated cholinesterase with benzoylcholine, which selectively blocked the sites. Then, they treated it with a high level an unlabeled DFP, thus phosphorylating all possible amino acids not on the sites. Then they dialyzed off the benzoylcholine and surplus DFP, and treated with labeled DFP, which only had the active centers left to phosphorylate.

Reversible inhibitors such as eserine also protect cholinesterase from inhibition of organophosphates. Burgen[23] showed that at a whole series of eserine concentrations there was protection against TEPP (1.3×10^{-8} M) (Fig. 3.9), and that "more or less efficient" protection was given by neostigmine, choline, and carbamoyl choline.

Koelle[69] has shown that two bis quaternary reversible inhibitors of cholinesterase, N,N'-bis (2-diethylaminoethyl) oxamide bis-2-chlorobenzyl chloride ("ambenonium chloride") and its 2-methoxybenzyl analog, were very effective in protecting cholinesterase from DFP inhibition: thus, at an ambenonium level of 8×10^{-7} M which usually inhibited cholinesterase by 98%, there was 92% protection against 10^{-4} M DFP; while at 9×10^{-9}

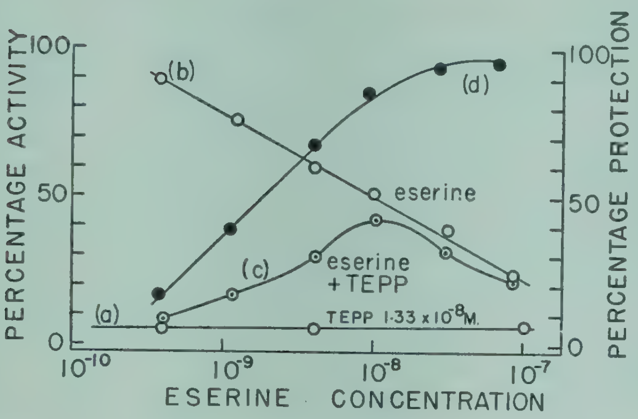
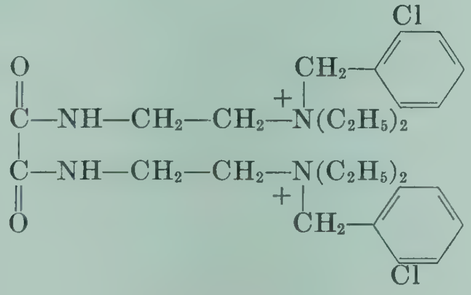


FIG. 3.9. Protection of erythrocyte cholinesterase by eserine against TEPP. (*a*) TEPP incubated for 20 minutes. (*b*) Eserine incubated for 20 minutes. (*c*) Eserine for 20 minutes followed by TEPP for 20 minutes. (*d*) % protection against TEPP by eserine. From Burgen.[23]

M ambenonium, the usual cholinesterase inhibition was 20% and there was 28% protection against DFP.



Ambenonium

The local anaesthetic procaine has been shown to antagonize Tabun inhibition of a number of esterases.[11] Alcohols have a protective effect,[93] and because of this, the common practice of adding organophosphates in ethanolic solution to the cholinesterase, in the course of determination of anticholinesterase activity, can lead to misleading results.[80] Another convenient solvent, propylene glycol, also has a protective effect.[80]

The amino acids have, in general, negligible protective effect, except in the case of catechol derivatives, which probably produce their effects by reacting with uncombined inhibitor, and so reduce the amount available for cholinesterase.[19]

Reversal of Inhibition (Reactivation)

(*a*) *Spontaneous.* It has been stated above that the organophosphates inhibit cholinesterase "irreversibly." Now it is generally true that dialysis, dilution, and washing are ineffective in restoring phosphorylated cholinesterase to activity. But if the inhibited enzyme is allowed to stand, it slowly recovers its activity, and the rate of recovery is characteristic: (1) of the enzyme, and (2) of the alkyl substituents of the organophosphate. Examples are given in Fig. 3.1. It is evident that reversal of dimethyl-phosphorylated cholinesterase is an important factor even under normal experimental conditions. Under the same conditions the reversal of diethyl-phosphorylated cholinesterase is small;[5] but reversal does occur in long periods. Thus, Wilson[96] has shown recovery of TEPP-inhibited electric eel cholinesterase, to the extent of 45% in 28 days at 7°C. (pH 7); Hobbiger[56] found recovery of TEPP-inhibited cholinesterase from various sources, from an initial 90% to a final level of 40% (approximate values), following incubation at 30°C. for 72 hours. The reversal of isopropyl phosphorylcholinesterase is slow enough to be negligible under practically any conditions.

The reversal rate depends not only upon the inhibitor, but (as would be expected) on the enzyme type. For any given inhibitor, pseudocholinesterase recovers more rapidly than true cholinesterase.[34] Davison[33] has shown that the spontaneous recovery of rat-brain pseudocholinesterase is extremely slow in comparison with the recovery of other pseudocholinesterases (serum, jejunum, heart, and diaphragm.) This is evidence that there are two forms of pseudocholinesterase, although there are no differences in behavior towards substrates or inhibitors. Later,[34] Davison noted an astonishing variation among species; the half-life for diethyl-phosphorylated serum pseudocholinesterase reversal in five species varied from 2.2 hours in the hen to 730 hours in man.

As described above in the proof that inhibition is a phosphorylative process, the spontaneous reversal rate is characteristic only of the dialkoxy part of the phosphate (page 74 and Fig. 3.1). For rabbit erythrocyte cholinesterase Aldridge[6] found that the order of reversal rate was: methyl > ethyl > isopropyl. In several rat pseudocholinesterases, however, Davison[34] showed that the diethyl phosphorylated enzyme recovers rapidly (the half-life at pH 7.8, 37°C. was 5 hours) but neither the dimethyl nor diisopropyl phosphorylated enzyme recovered to half activity in 200 hours. For human serum cholinesterase, Hobbiger[56] reported no reactivation of the diethyl phosphorylated enzyme. But Mengle and O'Brien[72] using Aldridge's technique for removing surplus inhibitor, found for human serum cholinesterase that 20–50% recovered in 24 hours from diethyl or dimethyl phosphorylation, and 2–7% recovered from diisopropyl phosphorylation (37.5°C.).

Reactivation is temperature-dependent, since it is a normal chemical reaction, involving hydrolysis of the phosphoryl enzyme to give a phosphate plus enzyme. The energy of activation is 14,400 cal. mole^{-1} for dimethyl phosphorylcholinesterase from rabbit erythrocytes.[5]

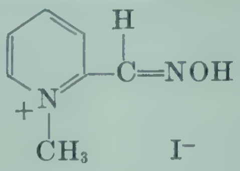
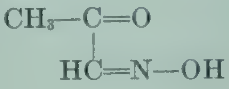
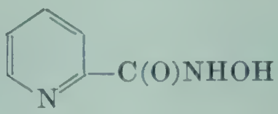
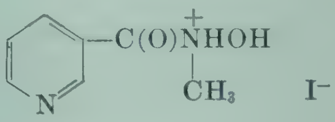
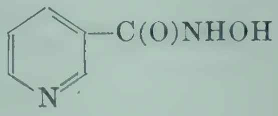
In 1958, Asperen and Dekhuijzen[10] reported a remarkable difference between insect and mammalian cholinesterase. Following *in vitro* inhibition with DDVP (a dimethyl phosphate), housefly head cholinesterase showed no recovery in nearly 3 hours, even though, under the same conditions, mouse brain enzyme recovered from 70% to 30% inhibition. This important observation was confirmed and extended to TEPP, DFP, paraoxon, and malaoxon by Mengle and O'Brien, who showed also that if housefly cholinesterase was inhibited *in vivo*, the enzyme would recover if the flies lived, but did not recover at all if the flies were homogenized.[72] This paradox was tentatively resolved by the following observation: when cholinesterase was inhibited *in vitro* by any of four phosphates, and then a fresh homogenate of whole flies or fly heads was added, considerable reactivation occurred. It was suggested that a highly labile reactivating factor is present in living flies.

A naturally occurring reactivating factor in mammalian sera was found by Neubert *et al.*[77] In this case, however, it was heat stable. It was also ultrafiltrable. Its presence accounted for the relatively rapid recovery of serum cholinesterase after organophosphate poisoning.

(*b*) *Induced.* It is obviously important to have antidotes for such toxic compounds as the organophosphates, whether they be regarded as potential warfare agents or simply as insecticides. A great deal of time has been devoted to a search for them, on both sides of the Atlantic. Until the last few years, the only (published) antidote was atropine (sometimes used with magnesium salt), whose action depends upon antagonizing the effect of the excess acetylcholine resulting from the inhibition of cholinesterase. An alternative approach was to seek a compound which would attack the inhibited cholinesterase and dephosphorylate it.

The reversal of inhibition involves a hydrolysis which yields (in the case of dialkyl phosphate inhibition) dialkyl phosphoric acid and the active enzyme. The hydrolysis of phosphate esters is usually catalyzed by hydroxyl ion, a weak nucleophilic reagent. It occurred to several workers that a stronger nucleophilic agent than water might accelerate this hydrolysis, and thus hasten the recovery of organophosphate-inhibited cholinesterase. Wilson has published a series of papers in which he has used increasingly better nucleophilic reagents, starting with hydroxylamine,[96] and continuing with derivatives of it. The most striking effect was produced when the reactivating group was attached to a residue containing a quaternary nitrogen group, at a distance from the reactivating group comparable to the distance between the anionic and esteratic sites of cholinesterase. Presumably, this conformation aids in the affinity of the molecule for the active center of the enzyme. The compound referred to is known as 2-PAM, or pyridine-2-

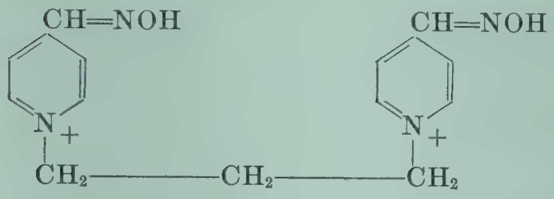
TABLE 3.6. AGENTS THAT REACTIVATE INHIBITED CHOLINESTERASE[a]

Agent	Structure	Reference
Pyridine-2-aldoxime methiodide (2-PAM)		101
monoisonitrosoacetone (MINA)	 $CH_3-C(=O)-HC=N-OH$	27
Picolinohydroxamic acid		102
Nicotinhydroxamic acid methiodide		104
Nicotinhydroxamic acid		103
Acethydroxamic acid	$CH_3C(O)NHOH$	103
Hydroxylamine	NH_2OH	103

[a] The compounds are listed in decreasing order of excellence.

aldoxime methiodide. Its structure, along with that of other reactivators, is shown in Table 3.6.

It is of interest that this compound was announced in 1955 independently and almost simultaneously by Wilson and Ginsburg[101] in the U.S.A., and by Childs *et al.*[27] in England. The potency is such that "it may reactivate 80% of alkyl phosphate inhibited enzyme in 10^{-5} *M* concentration within 1 minute."[68] However, in 1958 Hobbiger *et al.*[60] reported some new compounds which were up to 22 times more potent than 2-PAM in reactivating inhibited cholinesterase (such compounds had been described earlier, in 1958, by Poziomek *et al.*;[84] their superior reactivating power was mentioned, but the emphasis was on their therapeutic use in organophosphate poisoning). These resembled two units of 2-PAM (or, better still, 4-PAM) joined at their quaternary nitrogen groups by a carbon chain, optimally 3 carbons long.



1,3-bis(N-Pyridinium-4-aldoxime)propane

The regeneration of phosphorylated cholinesterase by oximes is strongly dependent upon pH, as shown in Figure 3.10.

Perhaps it has not been strongly enough stressed by many workers that most of these reactivating compounds also catalyze the nonenzymic hydrolysis of many organophosphates[51] (Table 2.9) and there are certain conditions under which this catalysis could be a factor in determining the extent of cholinesterase inhibition. This catalyzed hydrolysis is dealt with in Chapter 2 (pages 43–45).

The kinetics of the reactivation process by oximes has been studied by Davies and Green,[32] who have showed that the reaction follows second-order kinetics; the rate of reactivation at any moment depends upon the oxime concentration and the amount of enzyme still unreactivated

$$d[E]/dt = k[E_\infty - E][\text{oxime}]$$

where $[E]$ is the concentration of reactivated enzyme and $[E_\infty]$ is the total reactivable enzyme concentration. They found for human erythrocyte cholinesterase, k values of about 22.1 mole^{-1} for sarin and 7 for TEPP. The reactivator was monoisonitrosoacetone. The effect of temperature and pH

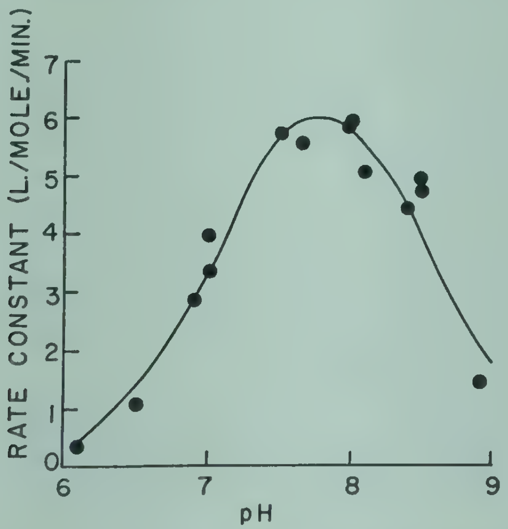


Fig. 3.10. Reactivation of diethyl-phosphorylated cholinesterase by MINA at different pH values. From Davies and Green.[32]

was also studied for these reactivators. A distinct optimum was noted at about pH 7.8 (as was true in Fig. 3.10). From the temperature variation studies were calculated the activation energies for the reactivation: they were about 11.5 kcal. mole^{-1} for both TEPP and sarin reactivation by diisonitrosoacetone. These authors also reported the effectiveness of several oximes and hydroxamic acid in reactivating variously inhibited erythrocyte cholinesterases.

Further kinetic studies by Green and Smith[49, 50] have given evidence that in the course of reactivation, both by 2-oxoaldoximes [RC(O)CH=NHOH] and 2-pyridinealdoxime methiodide, a complex between reactivator and inhibited enzyme is first formed. In the 2-oxoaldoximes it is likely that the complex is with the carbonyl group, since variations in the oxime's alkyl group (R) are not important in determining activity.

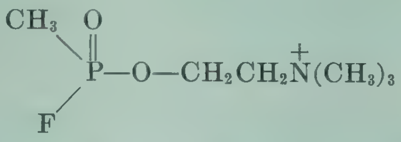
Reactivation of serum cholinesterase has also been demonstrated by Wilson,[99] using TEPP or DFP-inhibited cholinesterase and reactivating with hydroxylamine, pyridine, and hydroxamic acids. Quaternary-containing agents were better than their tertiary analogs, as for true cholinesterase, showing that the anionic site of pseudocholinesterase is still functional in the phosphorylated enzyme.

Wilson[97] has pointed out that those hydroxylamine derivatives that contain a quaternary nitrogen are 20–100 times better at reactivating diethyl than diisopropyl phosphorylated cholinesterase; but those that do not have a quaternary nitrogen are only 2–9 times better. He attributes this to the "promoting" activity conferred by the quaternary group, as a result of a binding to the anionic site of the cholinesterase. This promotion is less effective for the diisopropyl phosphorylated enzyme because of steric hindrance of the anionic site by this bulky alkyl group. The dissociation constants which have been calculated by Green and Smith[50] for these two forms of phosphorylated cholinesterases with 2-PAM are a measure of this promotion effect, and give quantitative support to Wilson's suggestion. That 2-PAM binds to the anionic site of diethyl phosphorylated cholinesterase was further demonstrated by Green and Smith[48] by showing that cations such as NH_4^+ and choline greatly reduced the reactivation of TEPP-inhibited erythrocyte cholinesterase by 2-PAM, but did not reduce the reactivation by isonitrosoacetone.

A histochemical study[18] on the motor end-plate has suggested that if 2-PAM is allowed to stay in contact with cholinesterase previously inhibited (by TEPP) but now fully reactivated, the 2-PAM will proceed to inhibit the enzyme.

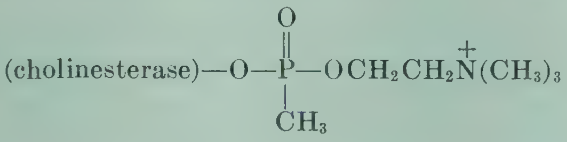
Reactivation of inhibited fly head cholinesterase is produced by 2-PAM and pyridine-2-aldoxime dodecaiodide (but not with diisonitrosoacetone), in spite of the absence of spontaneous reactivation.[72]

A remarkable case has been reported by Tammelin and Enander[92] of a phosphate which attacks cholinesterase to give an inhibited enzyme which cannot be reactivated by 2-PAM. The phosphate is:



cholinyl methylphosphonofluoridate

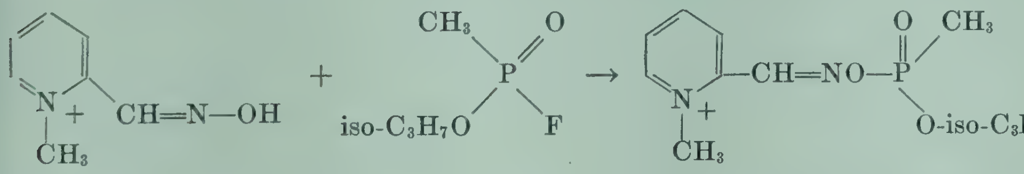
which the authors (using the Swedish system) call methylfluorophosphorylcholine. Apparently the phosphorylated enzyme is of this form:



$$(\text{cholinesterase})\text{—O—}\underset{\text{CH}_3}{\overset{\text{O}}{\text{P}}}\text{—OCH}_2\text{CH}_2\overset{+}{\text{N}}(\text{CH}_3)_3$$

and the quaternary nitrogen must combine with the anionic site; perhaps this bulky molecule adpressed to the enzyme prevents the attack of the 2-PAM on the phosphorus.

A hitherto unsuspected factor in the action of the oxime reactivators was revealed by Hackley *et al.* in 1959.[52] They found that the oximes can react with certain organophosphates to produce an anticholinesterase which is more potent than the parent compound: for example, 2-PAM combined with sarin as follows:



O-(isopropyl methylphosphonyl) -2-formyl-1-methylpyridinium oxime

This product, which they abbreviate as 2-PPAM, was rather unstable, with half-life of 15–30 minutes at pH 7.4 and 30°C. The analog in the 4-position (i.e., para to the N) which they call 4-PPAM, was more stable (half-life 200 minutes at pH 7.4, 30°C.), was synthesized in an organic solvent, and also shown to be produced under more "physiological" conditions: an aqeous medium (pH 7.6), room temperature, 4-PAM at 10^{-4} M, and sarin at 10^{-2} M. This 4-PPAM was 1.4 times more active than sarin as an anticholinesterase.

The authors point out that if such reactions occur normally in the attempted reactivation of cholinesterase by oximes (*in vivo* and *in vitro*), then the observed effectiveness of reactivation is less than the maximum possible, and that one may hope for compounds as good as 2-PAM in reactivation, yet lacking the capacity to react with the phosphates to form potent anticholinesterases. It seems that in the case of the isonitroso compounds such as MINA (page 102), the phosphorylated oxime is extremely unstable, and an effective new inhibitor is therefore not formed.

"Aging"

It was first observed by Hobbiger in 1955,[58] that the extent of reactivation of organophosphate-inhibited serum cholinesterase that could be obtained with the dephosphorylating agent, nicotinhydroxamic acid methiodide, depended upon how long the enzyme and inhibitor had been left in contact (Fig. 3.11). The longer the inhibitor was in contact, the less the fraction of inhibited enzyme that could be recovered by subsequent treatment with the agent. Hobbiger therefore proposed that the phosphorylation of cholinesterase led initially to "phosphorylated enzyme I," which gradually changed to type II. Type I could be readily dephosphorylated by appropriate nucleophilic agents, but not type II. He suggested that a transphosphorylation was involved; at the moment let us, however, refer to this phenomenon as "aging." In Hobbiger's study only 10% of the diethyl phosphoryl cholinesterase was of type II if the inhibitor was in contact for 30 minutes, but more than 90% was type II if the contact period was 24 hours. The "aging" was faster with enzyme inhibited by diisopropyl phosphates, but in this case the behavior depended to some extent on other parts of the phosphate than the alkoxy groups.

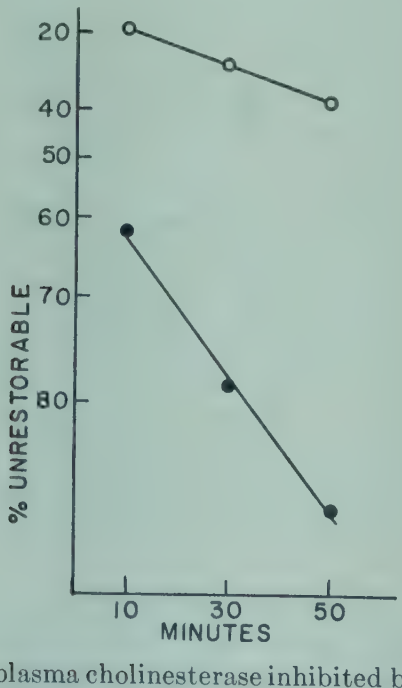


FIG. 3.11. "Aging" of plasma cholinesterase inhibited by DFP (○) or diisopropyl quinolinyl phosphate (●). *Ordinate*: % unrestorable by 0.1M nicotindroxamic acid methiodide. *Abscissa*: time of contact of inhibitor and enzyme. From Hobbiger.[58]

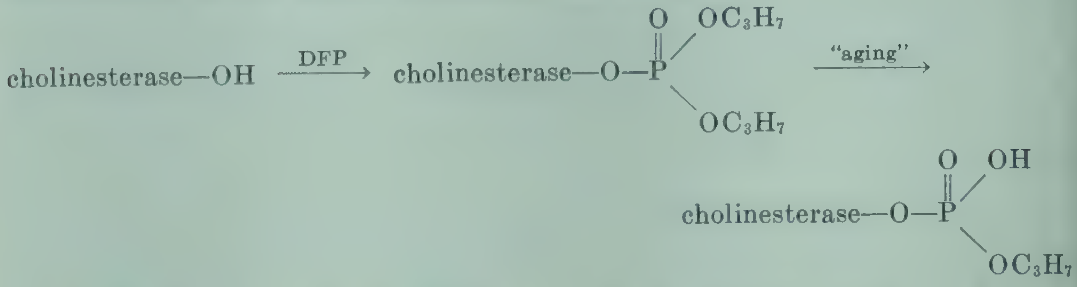
In later experiments,[59] Hobbiger found similar "aging" *in vivo* with brain and erythrocyte cholinesterase of mice. The "aging" rate depended upon the alkoxy substituents of the inhibitor, the half-time for the process being 36 hours with diethyl phosphates and 4 hours with diisopropyl phosphates.

In 1956, Davies and Green[32] reported similar observations with erythrocyte cholinesterase inhibited by TEPP or sarin *in vitro*. They noted that the rate of change from reactivatable to unreactivatable cholinesterase was negligible at 0°C.; followed first-order kinetics at 37°C.; and was pH dependent, being "markedly accelerated at lower pH and inhibited at higher pH." The rate of change was very slow with diethyl-phosphorylated cholinesterase, and fairly fast with the diisopropyl-phosphorylated and dimethyl-phosphorylated enzyme; these two latter forms changed at similar rates. These observations confirmed those made earlier by Wilson.[97]

On the basis of the assumption that the optimum pH for dephosphorylation by a reactivator was $\frac{1}{2}(pK_1 + pK_2)$, where pK_1 is the dissociation constant of the reactivator and pK_2 is that of the enzyme, Davies and Green conclude that the pK_a of the initially phosphorylated group in the cholinesterase is about 7.6. [In similar studies, Wilson *et al.*[103] had reported a pK_a of about 8.2, and identified it with the acid group of the esteratic site; they pointed out that phosphorylation would lower the normal pK_a of that group (i.e., 10.5) by reducing the nucleophilic influence of the neighboring basic group in the site.] They conclude that the ring nitrogen atom of histidine is the atom initially phosphorylated. Since serine phosphate is isolated from DFP-phosphorylated cholinesterase after degradation of the enzyme,[29, 87, 88] they conclude that the type I inhibited cholinesterase is histidine-phosphorylated, and that type II is serine phosphorylated (the serine phosphorylation being at the hydroxy group). If this is true, "aging" consists of transphosphorylation.

It had been suggested earlier by Wagner-Jauregg and Hackley[94] that histidine might be transiently phosphorylated by DFP in the course of cholinesterase inhibition, and that this would promote subsequent phosphorylation of another group. This suggestion was based on the finding that histidine (and even more so, its ring component, imidazole, alone) catalyzed DFP hydrolysis, probably by first forming diisopropyl phosphoryl histidine which promoted subsequent "phosphorylation of water," i.e., hydrolysis.

The possibility of an entirely different explanation for "aging" has been raised by Oosterbaan *et al.*,[83] in a piece of work which also throws doubt on the validity of using data on one esterase for conclusions about another esterase. They inhibited chymotrypsin, trypsin, and liver aliesterase with labeled DFP, then degraded the enzymes, and found that the serine was diisopropyl phosphorylated. But when the experiment was carried out with pseudocholinesterase (of unstated source), the serine was monoisopropyl phosphorylated. On this basis, they suggested that "aging" is caused by dealkylation of the dialkyl-phosphorylated enzyme:*



It should follow that chymotrypsin, etc. do not undergo "aging."

The occurrence of "aging" may account for the observations of Davison[34] that spontaneous recovery of rat cholinesterase *in vivo* after organophosphate treatment was never complete; and that the recovery proceeded briskly at first but came to a halt at 60% recovery. Davison's own suggestion was that there are two types of true cholinesterase; it seems more likely, now, that there is only one type, but that it may have two phosphorylated forms. Davison did, however, produce evidence that there was a pepsin-resistant fraction of true cholinesterase which recovered spontaneously only slowly if phosphorylated; but it is possible that pepsin altered the enzyme so that only type II phosphorylated cholinesterase could be produced on inhibition.

"Aging" of inhibited housefly head cholinesterase has been shown by Mengle and O'Brien.[72] However, in this case the variation in aging rate with the alkoxyl substituent of the phosphate was: methyl > ethyl > isopropyl. This observation precludes the possibility that the failure of phosphate-inhibited housefly cholinesterase to reactivate spontaneously (page 101) *in vitro* could be due to very rapid "aging." The "aging," in fact, proceeded steadily and slowly throughout the 24 hour period studied.

Selectivity for Different Cholinesterases

Many organophosphates inhibit acetyl- and pseudocholinesterase to a comparable extent. There are a number of important exceptions, however, some of which are given in Table 3.7. It is interesting to note the considerable variations, both in inhibitory concentrations and ratios of activities, that occur when different enzyme sources are used. Aldridge[4] has examined this variation in the case of two selective inhibitors for a number of cholinesterases (Table 3.8). An enormous variation with species was found, the inhibitory ratios for iso-OMPA (for instance), varying from 56 in man to 13,200 in the dog. Aldridge[4, 6] has suggested that the inhibitor activity ratios observed are comparable with the substrate activity ratios obtained

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* This hypothesis has been virtually proved for DFP and pseudocholinesterase by Berends *et al.*[15a]

TABLE 3.7. SELECTIVE INHIBITION BETWEEN TRUE AND PSEUDOCHOLINESTERASE[a]

Inhibitor	Aldridge[4]			Davison[34]		
	pI_{50} True	pI_{50} Pseudo	I_{50} True / I_{50} Pseudo	pI_{50} True	pI_{50} Pseudo	I_{50} True / I_{50} Pseudo
Dimethyl *p*-nitrophenyl phosphate	6.27	6.10	0.67	7.40	5.19	0.006
Diethyl *p*-nitrophenyl phosphate (paraoxon)	6.38	6.85	2.9	7.80	7.58	0.6
Diisopropyl *p*-nitrophenyl phosphate	5.50	6.50	10.0	6.49	7.15	4.5
Tetramethyl phosphorodiamidic fluoride	1.96	3.31	22	—	—	—
N,*N*′-diisopropyl phosphorodiamidic fluoride	3.82	7.42	3950	4.35	6.74	254
Diisopropyl phosphorofluoridate (DFP)	5.75	8.18	270	6.14	7.8	45
Di-*n*-propyl phosphorofluoridate	—	—	—	7.26	8.0	5.5
Diethyl phosphoric anhydride (TEPP)	6.52	8.39	73	7.85	7.92	1.2
N,*N*′-diisopropyl phosphorodiamidic anhydride (iso OMPA)	2.51	6.48	9400	3.57	6.34	590
Diisopropyl phosphoric anhydride	5.96	8.68	520	—	—	—

[a] *Results of:* Aldridge, horse erythrocyte (true cholinesterase) and serum (pseudocholinesterase); Davison, rat brain (true cholinesterase) or heart (pseudocholinesterase), 30 minutes, 37° C.

with the corresponding choline esters, e.g., comparing the substrate selectivity of the choline ester of $(CH_3)_2CHC(O)OH$ with the inhibitory selectivity of derivatives of $(CH_3)_2NP(O)(OH)_2$, but the data are not very convincing.

The selectivity for pseudocholinesterase of *N*,*N*′-diisopropyl phosphorodiamidic anhydride has been described in detail by Austin and Berry.[14] Qualitative reports of selective inhibition concern the oxygen analog of Guthion[35] and the *cis* isomer of Phosdrin,[26] both of which are specific for acetylcholinesterase.

Casida[25] studied the variation of inhibition of insect esterases by a fixed level (10^{-7}) of TEPP, and found a considerable variation in the 18 species tested, ranging from 1 to 93 % inhibition when acetylcholinesterase was the substrate. However, there was a considerable variation between species

TABLE 3.8. VARIATION OF SELECTIVITY OF TWO ORGANOPHOSPHATES WITH DIFFERENT MAMMALS[a]

Species	Iso-OMPA			Mipafox		
	pI_{50} True cholinesterase	pI_{50} Pseudo cholinesterase	$\frac{I_{50}\text{ True}}{I_{50}\text{ Pseudo}}$	pI_{50} True cholinesterase	pI_{50} Pseudo cholinesterase	$\frac{I_{50}\text{ True}}{I_{50}\text{ Pseudo}}$
Horse	2.47	6.52	11,300	3.80	7.42	4,200
Human	3.52	5.27	56	4.66	6.41	56
Guinea-pig	2.34	5.89	3,600	4.21	6.51	200
Dog	2.42	6.54	13,200	4.06	7.29	1,700
Rat	3.60	6.32	530	4.22	6.75	340
Sheep	3.20	—	—	4.41	—	—

[a] Results of Aldridge.[4]

in the substrate pattern of the preparations, so the results cannot be reliably attributed to variations in the property of acetylcholinesterase in the different species.

Conclusion

Now we have considered the events that occur when we can arrange that inhibitor and enzyme shall meet freely, in solution, and in the absence of interfering systems. Next we must survey the numerous enzymes that can profoundly modify the properties of the organophosphates which they can attack. Then we must face the appalling complexities when organophosphate, modifying enzymes, and target enzymes come together in the body, a multiphase heterogeneous dynamic engine. The facts described in the present chapter will then constitute but one part of the knowledge we shall need to account for those *in vivo* events.

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CHAPTER 4

Enzymic Degradation and Activation *in Vitro*

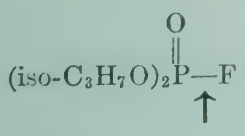
Probably all organophosphates can be degraded in the animal body, and in some cases the degradation is fairly rapid. Studies of these degradations *in vitro* have been more extensive than those *in vivo*, primarily because one can use high organophosphate concentrations that would soon kill the intact animal. Consequently tracer-labeled compounds are not needed. Also one can purify isolated enzyme systems and thereby avoid the experimental problems that arise when an organophosphate is attacked by several enzymes simultaneously.

Most of the economically useful organophosphates, as apart from the warfare agents, have to be "activated": they are very poor anticholinesterases until they are oxidized by certain enzyme systems. Whenever one finds a compound which is a poor anticholinesterase *in vitro* but which kills by an anticholinesterase action, one may suspect that activation is important. Unfortunately our knowledge of activating systems *in vitro* is very deficient, in part because of the complex nature of these systems as compared to the simple hydrolases which accomplish degradation.

A proper understanding of these processes is obviously a prerequisite for predicting the effects of new compounds upon animals. Whether an animal lives or dies when treated with an organophosphate is to a large extent an expression of the activating and degrading enzymes in the animal. We need to know for every species of interest both the specificity and the activities of these enzymes and their distribution in the various tissues.

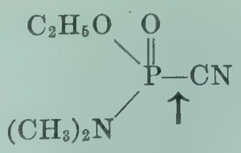
Degradation

Most organophosphates may be degraded by phosphatases, a class of enzyme which embraces several different kinds of hydrolase. The most studied *in vitro* are those which hydrolyze the warfare agents DFP, tabun, and sarin.

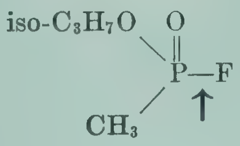


$$(\text{iso-}C_3H_7O)_2\overset{\overset{\displaystyle O}{\|}}{P}\underset{\uparrow}{-}F$$

DPF

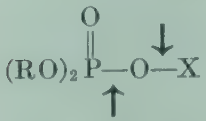


Tabun

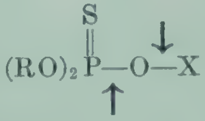


Sarin

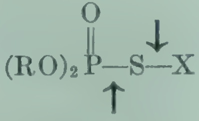
Of great practical interest are the phosphatases which attack phosphorus esters and thioesters:



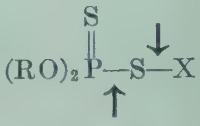
$(RO)_2\overset{\overset{O}{\|}}{P}\underset{\uparrow}{—}O\overset{\downarrow}{—}X$



$(RO)_2\overset{\overset{S}{\|}}{P}\underset{\uparrow}{—}O\overset{\downarrow}{—}X$

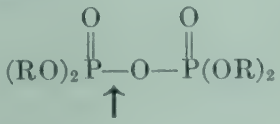


$(RO)_2\overset{\overset{O}{\|}}{P}\underset{\uparrow}{—}S\overset{\downarrow}{—}X$

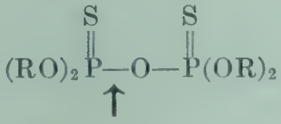


$(RO)_2\overset{\overset{S}{\|}}{P}\underset{\uparrow}{—}S\overset{\downarrow}{—}X$

Not all these hydrolyses have been demonstrated, but they are theoretically possible. The third major class of phosphatases attack symmetrical anhydrides, e.g.,



$(RO)_2\overset{\overset{O}{\|}}{P}\underset{\uparrow}{—}O—\overset{\overset{O}{\|}}{P}(OR)_2$

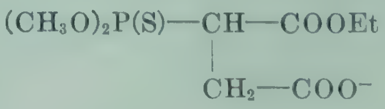


$(RO)_2\overset{\overset{S}{\|}}{P}\underset{\uparrow}{—}O—\overset{\overset{S}{\|}}{P}(OR)_2$

One might anticipate that there would be at least three discrete classes of enzymes catalyzing these three major classes of hydrolysis. In fact there is a good deal of overlap in activity. Unfortunately the excellent work that has been done on these enzymes has revealed a terrible complexity, so that one cannot yet tabulate a series of separate enzymes with known substrate preferences and known activities in the different species and their various organs. When such a table is available, it will be a tremendous assistance in attempts to predict the toxicity of new compounds.

Organophosphates containing carboxyester (COOR) or carboxyamide ($CONR_2$) groups are hydrolyzed by mammals primarily at these groups by carboxyesterases and carboxyamides, respectively. Consequently, in such compounds (e.g., malathion and dimethoate) phosphatase hydrolysis is less important. However, in plants and insects, *in vivo* studies show that carboxyesterases and carboxyamides are less important, and phosphatase action is correspondingly more important.

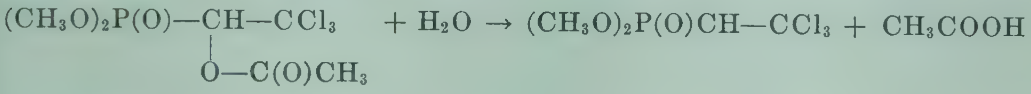
The vast majority of the phosphorus-containing products of organophosphate hydrolysis are anionic except in acid conditions, e.g., from parathion hydrolysis one obtains principally $(C_2H_5O)_2P(S)O^-$, from malathion, principally malathion acid



$$\begin{array}{l} (CH_3O)_2P(S)—CH—COOEt \\ | \\ CH_2—COO^- \end{array}$$

Consequently, these products are always ineffective anticholinesterases due to the marked nucleophilic effect of the anionic charge. Therefore, hydrolysis almost invariably detoxifies the compound completely. If one is interested exclusively in the toxic properties of an organophosphate and its metabolites, it is usually possible to follow the detoxification process by following the appearance of ionic phosphorus, i.e., phosphorus-containing material which cannot be extracted by (say) chloroform from water. The further degradation of these primary hydrolysis products is not of toxicological significance.

Exceptions to the rule that hydrolysis equals degradation may occur if the molecule has an ester link so arranged that on hydrolysis the alcoholic hydroxyl is on the phosphorus-containing moiety, e.g., acetyl Dipterex



$$(CH_3O)_2P(O)-\underset{\displaystyle O-C(O)CH_3}{\underset{|}{CH}}-CCl_3 + H_2O \rightarrow (CH_3O)_2P(O)CH-CCl_3 + CH_3COOH$$

Acetyl Dipterex

Dipterex

In this case hydrolysis in fact enhances the anticholinesterase potency of the molecule.[8]

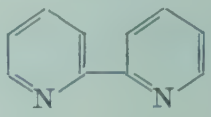
Phosphatase Action

"DFP-ase"

The first report on enzymic degradation of an organophosphate was made by Mazur in 1946.[52] He showed that DFP was hydrolyzed by homogenates of numerous tissues of the human and the rabbit. The order of activity of tissues was: liver, kidney, small intestine, plasma, lung, heart, brain, muscle, and red cells. This order was based on a fixed tissue concentration. If one based the data upon nitrogen content, kidney was more active than liver, and red cells more active than muscle. Plasma and liver also hydrolyzed three analogs of DFP: the dimethyl, diethyl, and methyl ethyl.

Mazur obtained from rabbit kidney by ethanol precipitation a preparation 13 times more active than the kidney homogenate. It was sensitive to Cu^{++} and also Hg^{++}, which caused severe inhibition even at $10^{-5}\ M$ (a paradoxical activation, to 240% of normal activity, was noted with $6 \times 10^{-6}\ M\ Hg^{++}$).

Seven years later, Mounter and his associates embarked on their extensive studies on "DFP-ase." First they made a purer enzyme preparation than Mazur had made (by precipitation with acid and ethanol) which was 70 times more active than the hog kidney source.[65] Using this pure preparation, they observed the following properties: (*a*) Co^{++} and particularly Mn^{++} activated the enzyme strongly (e.g., four-fold for $10^{-3}\ M\ Mn^{++}$); (*b*) Mg^{++} activated the enzyme weakly; and (*c*) the ten other divalent or trivalent ions inhibited the enzyme strongly. Other properties of the enzyme were established (e.g., effect of pH and substrate concentration) and activation by many nitrogenous compounds, including 7 amino acids, was noted. Histidine was the most effective amino acid, but 2,2′-dipyridyl was the most effective compound, particularly in the presence of Mn^{++}.



2,2′-Dipyridyl

Inhibition was found with compounds reacting with sulfhydryl groups (e.g., iodoacetate), carbonyl groups (e.g., phenylhydrazine), and metals (e.g., 8-hydroxyquinoline).

More extensive studies on activating agents[62] showed that only derivatives of imidazole or pyridine were highly effective, and that Mn^{++} was required for full effectiveness. These results are reminiscent of those described for the activation of nonenzymic hydrolysis (Chapter 2), where optimal results were obtained with copper and 2,2′-dipyridyl (purified DFP-ase is, however, inhibited strongly by copper). It would seem that optimal DFP hydrolysis requires a divalent metal and a complexing agent to facilitate the attack of OH^-, either directly or via DFP-ase. Perhaps in both cases the role of the metal complex is to combine with the DFP; this could activate hydrolysis either by making the P—F bond more susceptible, e.g., to nucleophilic attack, by an inductive mechanism or by presenting a sterically more favorable configuration to the attacking species. These two mechanisms could proceed with or without attachment of the metal complex to OH^- or enzyme prior to the hydrolysis. Mounter and Chanutin[61] suggest that an enzyme-metal-cofactor complex is formed, and that this attacks the DFP. On this hypothesis they formulate equations which they use to apply to their data, and evaluate the formation constants of metal-enzyme and metal-enzyme-activator. These constants yield predictions of the relative effectiveness of Co^{++} and Mn^{++} as activators of the enzymic hydrolysis of DFP which agree well with experimental results. Their hypothesis should perhaps be tentatively accepted. The possibility has, however, not been excluded that a DFP-metal-activator complex can be formed, much the same as was proposed for nonenzymic hydrolysis.

Kinetic analyses were also applied[59] to pH dependency and substrate effects. The Michaelis constant K_m varied markedly with Mn^{++} concentration, as one would expect from data given above (K_m, which is equal to the substrate concentration for half-maximal velocity, measures the affinity of substrate for enzyme). The pH optimum was independent of Mn^{++} at 8.5. The pK_a of the hypothetical active site was 7.2, identical with that of acetylcholinesterase. Mounter suggests that both may have histidine in their active sites.

Mounter *et al.*[64] examined numerous other tissues from human, rat, cat, guinea pig, pigeon, and turtle. All could hydrolyze DFP, and all were activated by Mn^{++} and Co^{++}. The liver was particularly active (Fig. 4.1). Certain tissues were activated more strongly by Co^{++} than by Mn^{++} (e.g., DIALKYLFLUOROPHOSPHATASES

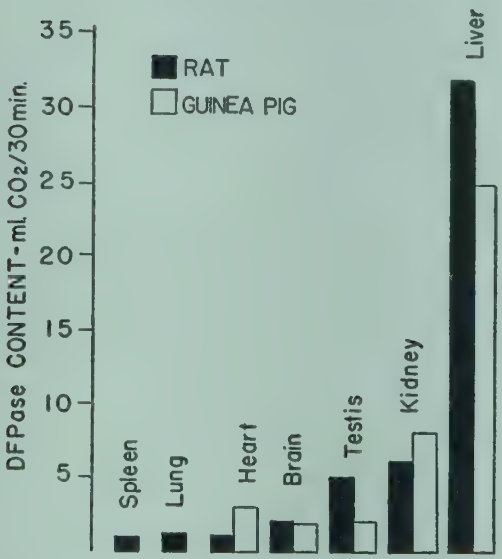


FIG. 4.1. "DFP-ase" content of homogenates of whole organs of the rat and guinea pig. From Mounter *et al.*[64]

mucosa, kidney, and liver of cat and kidney of man), whereas with others the reverse was true (e.g., liver, adrenal and spleen of man and testis of cat). This was evidence that more than one enzyme was involved; in fact, if the ratios of activity with Co^{++} as compared to Mn^{++} are taken to be fixed for a particular enzyme, there was evidence of a large number of different enzymes. However, the ratios usually were not widely different, and such data with crude tissue preparations may be affected by other factors than the nature of the enzyme.

A more intensive study then was carried out[57] on kidney and liver "DFP-ases." Three readily separable preparations were obtained from the hog and the rat. (*1*) A soluble enzyme (i.e., not precipitated at 30,000 × *g*, 60 minutes) of kidney was obtained that was activated by Co^{++}, more by Mn^{++}, and even more by Mn^{++} in the presence of histidine or dipyridyl. This preparation hydrolyzed DFP and its *n*-butyl analog, DBFB, at about equal rates. (*2*) A soluble enzyme of liver was obtained that was activated by Co^{++}, more by Mn^{++}, but on which histidine and dipyridyl had no effect. This enzyme hydrolyzed DFP and DBFP at equal rates. (*3*) An insoluble enzyme of liver was obtained that was activated by Ca^{++}, inhibited by Co^{++} and Mn^{++}, and unaffected by histidine or dipyridyl. This enzyme hydrolyzed DBFP about 10 times faster than DFP.

Differences were also noticed between species; thus the ratio of activities towards DBFP and DFP was about 1.3 in hog kidney, and about 1.0 in rat kidney. However, the activity of all preparations tested was extremely small (most results were between 0.2 and 5 μl. of CO_2 per 30 minutes, in

Warburg assays) and therefore only gross effects can be reliably accepted. Some insoluble kidney enzyme was present in the hog and the rat.

Mounter later suggested that DFP-ase was in fact an amino acid acylase,[58] since: (*1*) DFP-hydrolyzing activity paralleled hydrolysis of *N*-acetyl derivatives of valine, leucine, methionine, and alanine in a series of hog kidney fractions, and in the presence of several inhibitors; (*2*) the "two enzymes" had similar solubilities and pH optima, and responded similarly to temperature, ethanol, and metal ions; (*3*) mixed substrate experiments suggested a single enzyme; (*4*) the "two enzymes" were not separable by paper electrophoresis. Mounter therefore proposed that the normal function of DFP-ase was the hydrolysis and synthesis of amino acid derivatives. This conclusion may be incorrect in the light of the following observations of Cohen and Warringa.

Cohen and Warringa[25] purified hog kidney DFP-ase even further. By submitting the Mounter preparation to electrophoresis, they obtained a further four-fold purification. They also separated from the Mounter preparation by electrophoresis two unknown components, one green (which they call "G") and the other yellow ("Y"). These activated their purified DFP-ase slightly; but if either one was combined with Mn^{++}, it activated DFP-ase up to 25-fold — a greater effect than Mounter had obtained with his best organic activator, dipyridyl.

This purified preparation resembled the Mounter preparation in its susceptibility to inhibition and (qualitatively) to Mn^{++} activation. However, it showed zero activity towards acetylated amino acids. The authors therefore felt that Mounter's idea that DFP-ase is an amino acid acylase was incorrect, and that the acylase activity was due to an impurity. However, the possibility exists that the final purification modified the enzyme in some way with a resulting change in substrate specificity. In order to disprove this alternative, it would have to be shown that amino acid acylase activity was present in a fraction separable by electrophoresis from purified DFP-ase.

The substrate specificity of the highly purified DFP-ase was examined by Cohen and Warringa, and ten other organophosphates were hydrolyzed, most of them more vigorously than DFP. The following compounds were affected (A1–A10).

(A1) Ethyl methylphosphonofluoridate
(A2) *n*-Propyl methylphosphonofluoridate
(A3) 2,2-Dimethyl *n*-propylphosphonofluoridate
(A4) Isopropyl cyclohexylphosphonofluoridate
(A5) Cyclohexyl ethylphosphonofluoridate
(A6) Isopropyl methylphosphonofluoridate
(A7) Isopropyl isopropylphosphonofluoridate
(A8) Soman

(A9) Sarin
(A10) Tabun

The following compounds were unaffected (B1–B8).

(B1) Parathion
(B2) Schradan
(B3) ATP
(B4) Sodium pyrophosphate
(B5) Triacetin
(B6) Creatine phosphate
(B7) Acetylcholine
(B8) Butyrylcholine

The authors pointed out that the substrates are of three types: group (I) (DFP only) is a diester of phosphorofluoridic acid, group (II) compounds (A1–A9) are monoesters of phosphonofluoridic acid, and group (III) (tabun only) is an ester of a phosphoramidic acid.

$$\underset{\text{I}}{(RO)_2P(O)F} \qquad \underset{\text{II}}{(RO)R'P(O)F} \qquad \underset{\text{III}}{(RO)(R_2'N)P(O)F}$$

They found that in general, compounds of (I) or (III) were activated by Mn^{++}, those of (II) were not (there was a single exception: compound (A2) of the above table was of group (II) but was activated by Mn^{++}. Even greater complexities set in if activation by Mn^{++} + "G" was studied: all compounds except (A4), (A5), and (A8) were activated; and in this case the activation of DFP hydrolysis was vastly more potent than that of any other compound (e.g., up to twenty-ninefold, compared with eightfold for the rest of the other compounds).

These involved results seem at first to suggest an inhomogeneity of the purified preparation. However, if one considers that a complex of metal-activator-substrate-enzyme is probably formed, it is quite feasible that the metal-activator will complex differently with different substrates, even though the enzyme is identical.

Experiments carried out, using mixed substrates, suggested that only one enzyme was involved for hydrolysis of the following pairs: (*a*) DFP and (A4); (*b*) DFP and sarin; (*c*) DFP and (A7); (*d*) DFP and soman. However, DFP and tabun on this criterion seemed to be hydrolyzed by different enzymes (the criterion is this: that either, substrate A or substrate B, each 10^{-3} *M*, for example, should be hydrolyzed faster than a mixture of A and B, each 10^{-3} *M*, if only one enzyme is involved). If this is true, then even highly purified DFP-ase is inhomogeneous, and there really is a "tabun-ase" as distinct from a DFP-ase.

A variety of DFP-ases are to be found in microorganisms. Mounter *et al.*[60] studied 19 species, and found enormous variations in activity: the two micrococci studied had zero activity, whereas *Proteus vulgaris* had the greatest activity. At least 11 different patterns of behavior, with respect to the effects of Mn^{++}, Co^{++}, and Mg^{++}, were found in the different species:

this may be caused by 11 different enzymes, or more probably by different combinations of a lesser number. Histidine and 2,2′-dipyridyl had no effect upon any of these DFP-ases.

A more intensive study[66] on four organisms with 14 different substrates was made. The following compounds were hydrolyzed by all the organisms (C1–C8).

(C1) DFP
(C2) Ethyl analog of DFP
(C3) *n*-Butyl analog of DFP
(C4) TEPP
(C5) *n*-Propyl analog of TEPP
(C6) Isopropyl analog of TEPP
(C7) DDVP
(C8) Diethyl phosphorocyanidate

The following compounds were not appreciably hydrolyzed (D1–D7).

(D1) Dimefox
(D2) Schradan
(D3) Dipterex
(D4) Triethyl phosphate
(D5) Paraoxon
(D6) Parathion
(D7) Chlorthion

There was some evidence that a single enzyme was involved in the hydrolyses, since four inhibitors had comparable effects on the hydrolyses of several of the substrates; and mixed substrate studies showed less than additive effects. However, the inhibition data is only qualitatively acceptable, and the possibility of more than one enzyme is by no means excluded.

"Tabunase" and "Sarinase"

Augustinsson and Heimburger[12] have examined the enzymic hydrolysis of tabun, by the enzyme(s) which they call "tabunase" or "phosphorylphosphatase." It was present in the plasma of the eight mammals studied, and was very high in rabbit plasma. Other active tissues (in order) were spleen, adrenal, kidney, and liver. They considered that they had evidence of the identity of tabunase and DFP-ase, because of a comparable ratio of activities in the various tissues. However, the ratios were similar only in the adrenal cortex and medulla of the pig and the cow, and differed in the kidney of the pig and the cow, (Table 4.1). The data, on the contrary, prove that there cannot be only one enzyme in all these tissues. This conclusion is in line with Mounter's demonstration that there are a number of different DFP-hydrolyzing enzymes. It appears from Table 4.1 that the same tabun-splitting enzyme or possibly the same ratio of mixed enzymes is present in the adrenal cortex and the medulla of both species.

Detailed studies on the effect of metals upon tabun hydrolysis were made[10, 16] using the hog preparation that Mounter used for kidney DFP-ase, and a fraction of human serum. The results were complex (21 metals were examined), but the most important were: (*a*) Mn^{++} and Co^{++} activated the kidney enzyme and inhibited the serum enzyme; (*b*) Sr^{++} and

TABLE 4.1. RELATIVE ACTIVITIES TOWARD DFP AND TABUN OF ESTERASE PREPARATION[a]

Tissue	$\frac{\text{Hydrolysis of tabun}}{\text{Hydrolysis of DFP}}$
Cow kidney cortex	2.5
Cow adrenal cortex	1.5
Cow adrenal medulla	1.6
Pig kidney cortex	0.89
Pig adrenal cortex	1.6
Pig adrenal medulla	1.5

[a] Calculated from data of Augustinsson and Heimburger.[12]

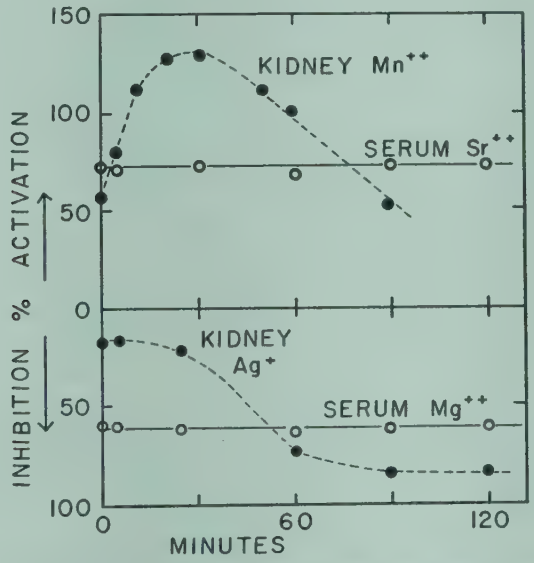


FIG. 4.2. Variation with time of the effects of salts upon "tabunase" from human serum or pig kidney. From Augustinsson and Heimburger.[16]

$Mn^{++} = 1.25 \times 10^{-3} M$ $Ag^{+} = 3.75 \times 10^{-3} M$ $Sr^{++} = 1.25 \times 10^{-3} M$

Ba^{++} activated the serum enzyme and inhibited the kidney enzyme; (*c*) most of the other ions, e.g., Ni^{++}, Pb^{++}, Cu^{++}, and Zn^{++} inhibited both kidney and serum enzymes, giving 50% inhibition at 10^{-3} to 10^{-4} *M*; (*d*) Hg^{++}, although an inhibitor of kidney enzyme at high concentrations, was an activator at low concentrations (as Mazur had shown for kidney DFP-ase).

The time effect with cations was odd (Fig. 4.2). The kidney and serum enzymes behaved quite differently. With the serum enzyme, a simple electrostatic effect was indicated and the effect was prompt and unchanging. With the kidney enzyme, a complex behavior was evident.

The hydrolysis of tabun and of sarin was examined by Adie and Tuba[2, 3] with a series of enzyme preparations. In rat serum, the two substrates showed identical Michaelis constants. With bovine plasma they had similar activation energies (sarin 15,200 cal. per mole; tabun 14,600) and thermal stabilities, and an almost identical dependence of activity upon pH. The ratio of activities towards sarin and tabun remained constant during a 1000-fold purification of the bovine plasma preparation. A study on four centrifugal fractions of monkey liver showed that each fraction contained an identical percentage of the total for sarin and for tabun. All these findings strongly suggest that a single enzyme degrades sarin and tabun.

However, a study of several tissues from 6 mammalian species showed a statistically significant variation in the ratio of activities towards the two substrates. The range was 0.9 ± 0.1 in rabbit brain to 4.8 ± 0.02 in rat liver (ratio of activities sarin:tabun). The figures for the ratios in the 6 plasmas are: 1.2, 2.1, 1.7, 1.1, 1.2, and 1.0. It seems quite possible that plasma contains only one enzyme for both substrates, and this enzyme is very similar in all species; but that in other tissues more than one enzyme is present. The plasma enzyme has been purified, giving a preparation 1250 times greater in activity than the (bovine) plasma.[1]

The distribution of sarin-splitting activity in four centrifugal fractions of the liver and kidney of five mammalian species was studied by Adie and Tuba.[3] In general, the order of activity was supernatant > microsomes > mitochondria > "nuclei." The "nuclei" (or rather nuclei plus debris plus unbroken cells) commonly had negligible activity, and the mitochondria never contained as much as one-fifth of the total activity. The pattern of distribution amongst the fractions showed no marked variation with species, nor was there a correlation between the total hydrolyzing activity and the resistance to sarin poisoning even though the mammals were selected with this in mind, i.e., rabbit, guinea pig, and monkey (susceptible), and rat and mouse (resistant).

Optical Specificity. Hoskin and Trick[48] showed in 1955 that rat serum hydrolyzed the toxic dextrorotatory isomer of tabun much more rapidly than the almost nontoxic levorotatory isomer. A study by Augustinsson,[11] in 1957, showed that the various tabun-hydrolyzing enzymes had different degrees of specificity for tabun optical isomers. Using purified preparations, the liver and kidney enzymes of the hog showed clear-cut differences, one isomer being hydrolyzed over ten times faster than the other (to be more precise, it was shown that pure tabun was hydrolyzed as if it contained two components of varying effectiveness as substrates, and it was assumed from Hoskin and Trick's work that the two components were the optical isomers). Purified human plasma enzyme, however, did not distinguish between the isomers; whole rabbit plasma showed a little specificity; whole rat plasma showed distinct specificity, but by no means as complete as did the hog kidney enzyme.

Other Phosphatases

Aldridge[4] examined serum esterases hydolyzing *p*-nitrophenyl acetate, propionate, and butyrate in 12 mammalian and avian species; these three substrates were hydrolyzed by various enzymes. He found marked differences in sensitivity of these enzymes to paraoxon, and on this basis classified the serum esterases as the "A-esterases," which are not inhibited by 10^{-8} *M* paraoxon, and the "B-esterases," which are inhibited by 10^{-8} *M* paraoxon. In general, the A-esterases hydrolyze *p*-nitrophenyl acetate faster than butyrate, and the B esterases the reverse is true (this is the basis for the terms "A" and "B"). Aldridge then showed[5] that A-esterase was unaffected by paraoxon because it hydrolyzed it; the hydrolysis of paraoxon by serum, moreover, was shown to be due entirely to A-esterase. Probably paraoxon is bound to the esteratic site of A- and B-esterases as well as cholinesterases; the A-esterase then hydrolyzes it as if it were a carbon acid ester, but the B-esterases and cholinesterase cannot carry out the hydrolysis, and remain blocked. Although the terms have in later years been used a little more widely, the initial definition reserves A-esterase for the enzyme in serum. The distribution of A-esterase in 9 mammalian species is shown in Fig. 4.3; there is about 16 times more in the rabbit than in any other species studies. A-esterase is not identical in all species: the ratio of activity towards phenyl acetate and paraoxon was: rabbit, 4; rat, 12; and horse, 19.

Aldridge[5] also studied the degradation of paraoxon by other tissues of rat (Fig. 4.4) and rabbit. In the rat, liver was most active; but in the rabbit the order of activity was serum, liver, heart, kidney, spleen, and muscle. It is noteworthy that the order for rat tissues given in Fig. 4.1 differs from that for DFP-ase.

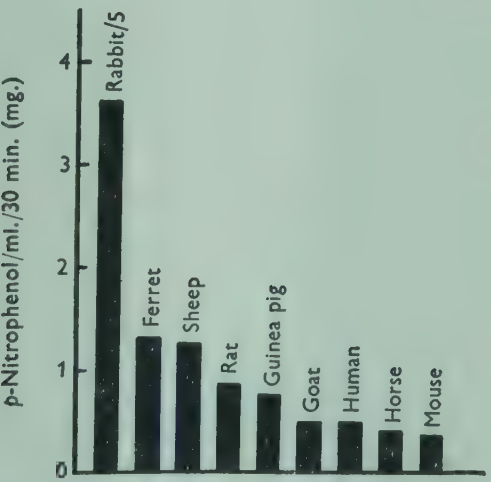


FIG. 4.3. Paraoxon-hydrolyzing activity of various sera. The value for rabbit has been reduced to one-fifth in the figure. From Aldridge.[5]

The hydrolyses of a number of organophosphates by rabbit serum were examined by Mounter.[56] He found that the following were hydrolyzed: DFP, TEPP, and paraoxon; the following were not: parathion, schradan, diethyl phosphoric acid, triethyl phosphate, triethyl phosphite, and diethyl ethylphosphonate. There was some evidence that one enzyme hydrolyzed the three organophosphates since a constant ratio of activities to the three substrates was maintained (*a*) throughout a number of procedures designed to degrade activity partially: heating, inhibition by metals, arsenite, etc., and (*b*) in various electrophoretic fractions. In five different animals, mixed substrate data gave confirmatory results. The tentative conclusion was drawn that there is only one A-esterase in rabbit serum.

Rabbit serum A-esterase reacts like liver-insoluble DFP-ase in that it is inhibited by Co^{++} and Mn^{++}, and thus differs from liver-soluble and kidney DFP-ase. Furthermore, kidney DFP-ase has no effect upon paraoxon.

Specificity studies were carried out by Augustinsson and Heimburger on serum enzymes, using (*a*) crude rabbit plasma and (*b*) a fraction of human serum, with the results shown in Table 4.2. A glance at the activity ratio for the different substrates will show that the two preparations are not identical. Experiments using mixed substrates suggested that in both preparations tabun, paraoxon, and DFP were hydrolyzed by a single enzyme, whereas TEPP was split by a different one.

The work so far described has in considerable part to be reconsidered in the light of two fine studies by Main.[50, 50a] He has made a highly purified (114×) preparation of "paraoxonase" from sheep serum, and also studied other serum degradations. Some conclusions were: (*a*) sheep serum "paraoxonase" does not hydrolyze TEPP; (*b*) sheep serum "paraoxonase" hydrolyzes DFP, a hydrolysis which is inhibited by manganese; (*c*) serum of

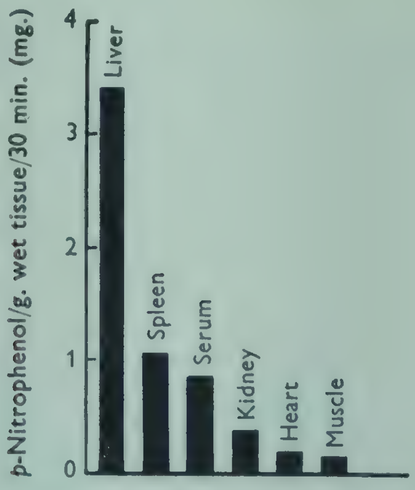
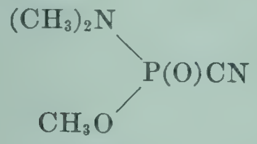
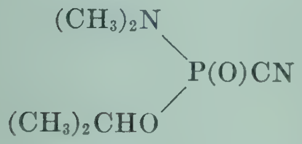
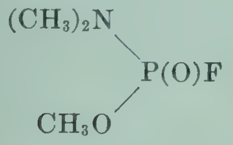


FIG. 4.4. Paraoxon-hydrolyzing activity of rat tissue homogenates. Activities of pancreas, brain, and submaxillary gland were undetectable. From Aldridge.[5]

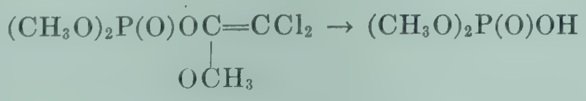
TABLE 4.2. HYDROLYSIS OF ORGANOPHOSPHATES BY SERUM ENZYMES[a]

Compound (usually 5×10^{-3} *M*)	Hydrolysis rate (μl. CO_2 per 30 minutes)		
	Rabbit plasma	Human serum fraction IV–I	Ratio
Tabun	315	170	1·9
	400	450	0·9
	62	79	0·8
	60	26	2·3
$[(CH_3)_2N]_2P(O)Cl$	—	0	—
$[(CH_3)_2N]_2P(O)F$	—	0	—
p-Nitrophenyl acetate	370	650	0·6
TEPP	331	43	7·7
DFP	179	132	1·4
Paraoxon	208	39	5·3
Chlorthion	0	0	—
Systox	0	0	—
Iso-Systox	0	0	—
Malathion	0	0	—
Diazinon	0	0	—

[a] Data of Augustinsson and Heimburger.[15]

sheep and horse, but not rabbit, contains also another, manganese-stimulated, DFP-ase; (*d*) most of the tabun-hydrolyzing activity of sheep serum is due to "paraoxonase"; (*e*) "paraoxonase" of sheep serum hydrolyzes *p*-nitrophenyl acetate but not phenyl acetate; (*f*) there is an enzyme in sheep and hog serum which hydrolyzes *p*-nitrophenyl acetate but not paraoxon. The name "D-esterase" was proposed.

The hydrolysis of a vinyl phosphate, Chlorophan, has been described by Herrmann and Pulver.[46] The enzyme was present in plasma (5 species) and in liver, kidney, and lung of the dog.



$$\underset{\text{Chlorophan}}{(CH_3O)_2P(O)O\underset{\displaystyle OCH_3}{\underset{|}{C}}{=}CCl_2} \rightarrow (CH_3O)_2P(O)OH$$

Chlorophan

Cleavage Site

So far we have discussed phosphatic hydrolyses without considering where the molecule is cleaved. Mazur's DFP-ase in rabbit serum[52] produced about 0.7 moles of F^- for every mole of DFP hydrolyzed and a negligible amount of PO_4^{3-}. Although Mazur suggested that the cleavage was exclusively of P—F, there may have been substantial cleavage of one isopropyl link (e.g., one-third of the total hydrolysis); it is very likely that the ionized product would not undergo further cleavage to PO_4^{3-} because the difference between an ionized and un-ionized substrate is a radical one. Unless Mazur's fluoride recoveries were exceptionally low, we may take the data as proof that an isopropyl link was split, but cannot say whether this preceded, followed, or prevented the P—F split. Mounter and Dien[63] showed with hog kidney DFP-ase that about 0.97 mole of F^- was produced for each 2 moles of CO_2 evolved during enzymic hydrolysis (in a bicarbonate buffer) suggesting that, in this case, hydrolysis was exclusively of the P—F bond.

The tabunase of rabbit and horse plasma hydrolyzes tabun exclusively at the P—CN bond.[13] Sarin is hydrolyzed by rat serum exclusively at the P—F bond, and the resultant isopropyl methylphosphonate is not hydrolyzed at all.[47]

Although there is as yet no *in vitro* evidence, it should be pointed out at this stage that *in vivo* a number of compounds of the type $(RO)_2P(S)X$ are hydrolyzed at the R—O—P linkage (Chapter 5), and, therefore, presumably appropriate esterases will eventually be found *in vitro.*

Secondary Cleavage

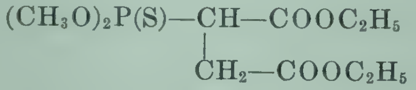
Major interest in hydrolysis is naturally centered on the primary cleavage because this constitutes a detoxification. However, in most cases, these primary products are susceptible to further alterations, as will be seen in Chapters 6 and 7 when the multiplicity of metabolites is seen after treating animals with a labeled organophosphate. A limited number of observations have been made of secondary reactions *in vitro.*

Binkley[18] has shown that the disodium salt of phosphorothioic acid is oxidized by kidney and liver homogenates of rats, yielding orthophosphoric acid. Forrest[39] has shown an extremely meager hydrolysis of *O*,*O*-diisopropyl phosphorodithioate by an *Aspergillus* preparation, using grams of enzyme, 0.1 *M* substrate, and 12 days of incubations.

A special case of phosphatase action occurs when the substrate is phosphorylated cholinesterase. In Chapter 2, it has been described how this "compound" slowly and spontaneously hydrolyzes, yielding a substituted phosphoric acid and active cholinesterase, and how this hydrolysis is accelerated by agents such as oximes. It seems that the hydrolysis can also be enzymically accelerated. Tabunase has been reported to reactivate cholinesterase that has been inhibited by tabun.[10, 14] Trypsin doubles the rate of recovery of pseudocholinesterase which has been inhibited by various (unstated) organophosphates.[28] Since trypsin degrades acetylcholinesterase, it cannot be used to reactivate this enzyme.

ESTERASE AND AMIDASE ACTION

Let us now consider examples of the more complex hydrolyses which occur when ester-linkage other than phosphatic ones are present. Examples that have been studied are:

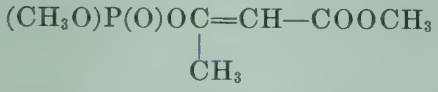


$$(CH_3O)_2P(S){-}\underset{\displaystyle CH_2{-}COOC_2H_5}{CH}{-}COOC_2H_5$$

Malathion

$(C_2H_5O)_2P(S)CH_2COOC_2H_5$

Acethion



$$(CH_3O)P(O)O\underset{\displaystyle CH_3}{C}{=}CH{-}COOCH_3$$

Phosdrin

$(CH_3O)_2P(S)SCH_2CONHCH_3$

Dimethoate

The carboxyester or carboxyamide link of such compounds is the most labile in mammals, which therefore hydrolyze them to the corresponding carboxyacids. Phosdrin may, however, be an exception.

The degradation of malathion and malaoxon by mammalian preparations has been studied in several laboratories. O'Brien[74] first showed that malaoxon was rapidly degraded by mouse tissues, particularly by liver, kidney, and lung. More extensive studies by Murphy and DuBois[67] in the rat, mouse, guinea pig, and dog showed considerable species variation in tissue activity (Table 4.3). Treatment of rats with EPN was shown to produce liver and serum with a markedly reduced capacity to degrade malaoxon: after 1.5 mg./kg. of EPN administered 1 hour before sacrifice, the degradation of malaoxon was inhibited 75% in liver, and 100% in serum.

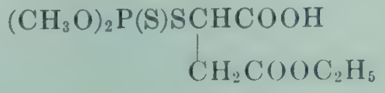
Cook *et al.*[26, 27] investigated malathion degradation in homogenates of

TABLE 4.3. MALAOXON DEGRADATION BY VARIOUS TISSUES[a]

	Rat	Mouse	Guinea pig	Dog
Liver	4·0	1·8	3·7	12·0
Kidney	0·7	0·5	0·2	1·2
Serum	1·0	1·8	0·2	0·1
Lung	0·8	0·6	0·2	1·2
Ileum	0·4	0·2	0·1	0·1
Spleen	0·1	0·03	0·2	0·03

[a] Micrograms malaoxon destroyed/milligrams tissue/10 minutes. Data of Murphy and DuBois.[67]

rat liver. They found (by paper chromatography) only one metabolite extracted by their technique, i.e., chloroform extraction of the neutral preparation. Subsequently, they showed that the single metabolite produced from acetone powders of rat liver was identical with a synthetic (crude) sample of a monocarboxyester of malathion which we may call "malathion monoacid."

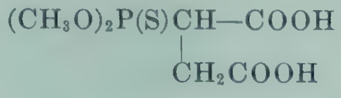


$$(CH_3O)_2P(S)S\underset{\displaystyle CH_2COOC_2H_5}{\underset{|}{C}}HCOOH$$

The identity was based on infrared, paper chromatographic, and partitioning data. The authors refer provisionally to the system responsible for this hydrolysis to the monoester as "malathionase." They observed chromatographically that malathionase was inhibited *in vitro* by a number of organophosphates at 4×10^{-4} *M*; partial inhibition was given by Thimet, Systox, Phosdrin, Diazinon, and methyl parathion, and complete inhibition by parathion.

They also examined the relationship between inhibitor concentration and malathionase inhibition more closely, and showed clearly that the P=O analogs of EPN, parathion, and Guthion (which they prepared by bromine oxidation) were vastly better malathionase inhibitors than their parent compounds. Their data for Systox, Phosdrin, and Dipterex, which without oxidation show anticholinesterase activity comparable to oxidized EPN, parathion, or Guthion, are surprisingly dependent upon procedure. With a colorimetric assay for malathion (Figs. 4 and 5 ref.[26]) and simultaneous addition of malathion plus inhibitor, these compounds were all very inferior even to unoxidized EPN. Thus, 4×10^{-4} *M* Phosdrin inhibited malathionase by about 40%. But using a manometric procedure, with ten minutes elapsing between adding inhibitor and malathion, Phosdrin was superior even to oxidized EPN (Fig. 9 ref.[26]), and about 4×10^{-7} *M* Phosdrin gave about 40% inhibition. The inhibition by paraoxon and methyl paraoxon of malathionase resembles (as one would expect) that of cholinesterase in that it is progressive.

The degradation of malathion by various rat tissues was studied later by Seume and O'Brien[83] using radioactive malathion. They found that all eleven tissues studied could degrade malathion—and liver was by no means the most active tissue; kidney was most effective. Ten hydrolysis products were separated in most tissues; about 85% of these were carboxyesterase products. The monoacid comprised about 35%, the diacid about 50%.



$$(CH_3O)_2P(S)\underset{\displaystyle CH_2COOH}{\underset{|}{C}}H\text{—}COOH$$

Malathion diacid

Treatment of the rats with EPN (or incubation of normal tissues with EPN *in vitro*) shifted the whole distribution of metabolites, so that up to 58% now appeared as phosphatase products, the most important (as much as 41%) being dimethyl phosphorothioate, $(CH_3O)_2PSOH$. This treatment also had the effect of sharply reducing the over-all hydrolysis, presumably by specifically inhibiting the carboxyesterase(s). This study serves to point up the dynamic nature of the degradation of a complex molecule: many routes are available and a competition exists amongst the different enzyme systems, a competition whose results can be radically rearranged by appropriate selective inhibitors. It is probably undesirable to use the term malathionase in view of the multiplicity of the enzymes involved. Personally, the author sees no objection to using terms like "X-ase" when X is degraded by a single enzyme, whose natural substrate is unknown, as long as the term is adopted provisionally. But such a term should be reserved for cases where the homogeneity of the degrading system and its nonidentity with known enzymes can be shown.

The hydrolysis of acethion by mouse-liver homogenates was studied by O'Brien *et al.*,[79] and the principal product was found to be the corresponding carboxylic acid ("acethion acid"), with small amounts of diethyl phosphorodithioate and a very polar unknown compound. Liver microsomes also hydrolyzed acethion, but, in this case, acethion acid was the sole product.

$$\underset{\text{Acethion}}{(C_2H_5O)_2P(S)SCH_2COOC_2H_5} \rightarrow \underset{\text{Acethion acid}}{(C_2H_5O)_2P(S)SCH_2COOH}$$

Insect Hydrolases

Very little information is available on the hydrolysis of organophosphates by insect tissues *in vitro*. Metcalf *et al.*[55] have shown that the "aromatic esterase" of the bee abdomen can hydrolyze several organophosphates: parathion, paraoxon, diethyl phenyl phosphate, diethyl *p*-tolyl phosphate and diethyl *p*-chlorophenyl phosphate. Experiments with mixed substrates suggested that parathion and *p*-nitrophenyl acetate were hydrolyzed by the same enzyme. Parathion was hydrolyzed faster than paraoxon (twice as fast in the experiment reported), in contrast to mammalian preparations in which only paraoxon is hydrolyzed.

Neither schradan nor its potent metabolite, hydroxymethyl schradan, were hydrolyzed by cockroach homogenates.[78] Malaoxon was degraded very slowly by whole cockroach preparations or by cockroach guts in contrast to the rapid degradation by mammalian preparations.[74] Acethion was degraded more rapidly than malaoxon by cockroach minces, but was entirely unaffected by fly minces.[79] Co-ral was not degraded by homogenates of housefly or by homogenates of third instar cattle grubs.[80]

Oxidative Degradation

Scaife and Campbell[82] have reported the only known case of direct oxidative degradation (an indirect oxidative degradation can occur with some rather hydrolyzable phosphorothionates and phosphoramidates which are rendered very susceptible to hydrolytic degradation after oxidation). The compound concerned was Amiton (whose oxalate salt is called Tetram).

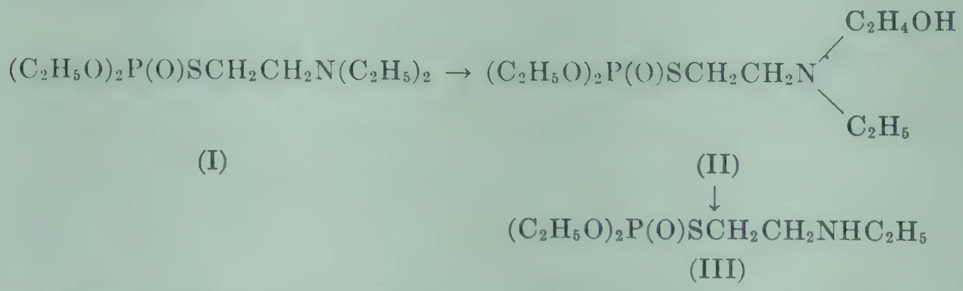
$$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$$

Amiton

This compound is a potent anticholinesterase requiring no activation. It was destroyed rapidly by liver homogenates, and virtually all of this destruction was catalyzed by the microsomes. The microsomes required oxygen, phosphate, and (unless prepared with a nicotinamide-containing medium) DPN. They did not require Mg^{++} or Ca^{++}, and DPNH was not better than DPN. An excess of DPN was inhibitory. The optimum pH was about 7.7.

The system was present only in liver, and in the rat its activity did not depend upon age or sex (in contrast to the Guthion-oxidizing system, see page 140). Activity was high in the rat, the rabbit, the mouse, and the guinea pig and about half as high in the pig, the dog, the cow, and the frog. Activity was absent in the cat and in the human. (This pattern differs somewhat from the pattern of schradan-activating activity.[72]) It resembled all the microsomal oxidation systems studied, so far, in being inhibited by SKF 525A, and resembled the Guthion-activating system by being inhibited by cytochrome C.

No clue was provided to the nature of the degradation product. By analogy with comparable microsomal reactions, one might anticipate oxidative de-ethylation:



$$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2 \rightarrow (C_2H_5O)_2P(O)SCH_2CH_2N\begin{matrix} \diagup C_2H_4OH \\ \diagdown C_2H_5 \end{matrix}$$

(I) (II)

$$\downarrow$$

$$(C_2H_5O)_2P(O)SCH_2CH_2NHC_2H_5$$

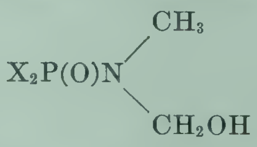
(III)

The hydroxyethyl form (II) is hypothetical, and probably only transient. It should be a substantially weaker base than (I), due to the electrophilic effect of the hydroxyl, but it is unlikely that this is enough to account for a severe reduction of activity (although in such compounds, the weaker the base, the poorer the anticholinesterase activity is likely to be, since only the protonated form binds to cholinesterase[76] and the % of protonation at a given pH is reduced by reduced basicity). In this area, all is guesswork, for the interesting variations of activity to be expected by modifying the alkylamine part of such compounds has never been explored.

It would also be premature to do more than note that there are striking similarities between this enzyme system and the phosphorothionate and phosphoramidate activating systems described below—and some equally striking dissimilarities. More research must precede a useful discussion.

Activation

Phosphoramidates, e.g., $X_2P(O)N(CH_3)_2$, and phosphorothionates, e.g., $(RO)_2P(S)OX$, are usually poor anticholinesterases *in vitro*. They are activated, i.e., converted into potent anticholinesterases, (*a*) *in vivo* in insects and mammals, (*b*) *in vitro* by liver slices or several insect whole-tissue preparations, and (*c*) *in vitro* by specially fortified liver homogenates. The product of phosphoramidate activation is the hydroxy alkyl derivative, e.g.,



The product of phosphorothionate oxidation is the phosphate,

$$(RO)_2P(O)OX$$

The activity of liver is due, principally, to its microsomes, which also catalyze a great number of other diverse reactions.

Tri-*o*-cresyl phosphate is also converted *in vivo* and by liver slices *in vitro* to a potent anticholinesterase whose identity is unknown.

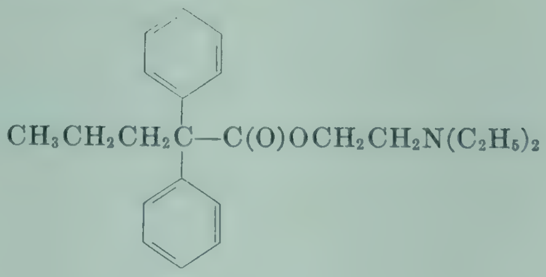
Phosphoramidate Oxidation by Mammalian Tissues

Schradan is a poor inhibitor of cholinesterase *in vitro* (e.g., 10^{-2} *M* is needed to give 50% inhibition of erythrocyte cholinesterase), yet seems to kill mammals by an anticholinesterase action, judging by the typical symptoms of salivation, lachrimation, fibrillation, etc., and the strong inhibition of blood cholinesterase in the poisoned animal.[42] Gardiner and Kilby,[42] therefore, suggested in 1950 that schradan was converted *in vivo* to a potent anticholinesterase and demonstrated this conversion in liver slices. DuBois *et al.*,[32] in 1950, independently made the same observation, and found brain, muscle, and kidney inactive. Mouse liver was only half as active as rat liver (but the sexes of the animals were not indicated; therefore, this result is uncertain). The activation product was unstable: standing for 48 hours (presumably in water or buffer) at room temperature destroyed it. The activation required air, being totally inhibited by anaerobic conditions.

Gardiner and Kilby[43] later reported on the properties of the inhibitor produced by liver slices. It was (unlike schradan) destroyed by heating for 10 minutes at 100°C; it was dialyzable; it was extractible from water into chloroform but not into less polar solvents such as petroleum ether, nitromethane, or nitrobenzene; it was destroyed completely in a few seconds by 0.033 *N* alkali; and it was destroyed partially in 30 seconds by .033 *N* acid.

Homogenization of liver destroys its capacity to activate schradan.[38] In 1954, Davison[30] published the important observation that rat-liver homogenates could activate schradan if they were fortified with magnesium, diphosphopyridine nucleotide (DPN), and nicotinamide (which prevents the liver from degrading DPN). Centrifugal fractionation of the homogenates showed that nuclei and mitochondria had no activating capacity, and a combination of microsomes plus supernatant gave the best activity. Liver homogenates from male mice were about twice as active as from females, which is in harmony with the observation that schradan is more toxic to the male than to the female mouse (LD_{50} : male 7, female 27 mg./kg.).

Schradan activation by microsomes plus soluble preparations was blocked by several compounds including sulfhydryl inhibitors (chloropicrin, iodoacetate), a carbonyl-binding agent (hydroxylamine), and a disrupter of oxidative phosphorylation (2,4-dinitrophenol). Cyanide was ineffective. The interesting agent SKF 525A was also inhibitory:



SKF 525A

SKF 525A is perhaps best known for its capacity to inhibit monoamine oxidase, the enzyme which degrades some physiologically interesting amines such as 5-hydroxytryptamine (serotonin).

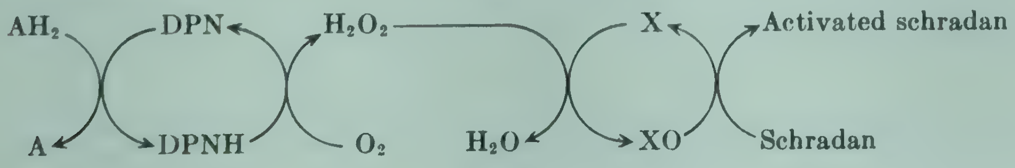
Davison considered that DPN was effective as such, without needing reduction to DPNH, because (*a*) his sample of DPNH was slightly less effective than DPN in effecting activation, and (*b*) oxidation of DPNH to DPN by microsomes plus supernatant was inhibited by schradan; if schradan activation utilized DPNH, one would perhaps expect DPNH oxidation to be enhanced by schradan.

Later work by O'Brien[72, 73] with the mouse confirmed Davison's observations on rat liver, except that it was shown that if DPNH was used in place of DPN, the supernatant fraction could be omitted. Presumably the function of the supernatant in Davison's system was to reduce the DPN to DPNH. Point (*b*), above, was explained by showing that DPNH oxidation by rabbit kidney is also inhibited by schradan, although this preparations was entirely ineffective in activating schradan. Furthermore, the amount of schradan activated was very small compared with the total DPNH utilized by a microsome preparation. In short, schradan activation utilizes DPNH, but this utilization accounts for only a small fraction of the DPNH consumed by microsomes. As for point (*a*), above, the explanation may lie in the impurity of the pyridine nucleotides employed by Davison. This may also account for another disagreement: Davison reported that crude TPN (triphosphopyridine nucleotide) or TPNH was ineffective, but O'Brien found TPN a little better than DPN in a microsome plus soluble preparation.

In the last few years an astonishing variety of oxidative reactions has been shown to be catalyzed by liver microsomes with DPNH or TPNH.[19] Usually, there is a specific requirement for one or other pyridine nucleotide, but the activation of schradan (and of the phosphorothionates, discussed below) appears to be an exception in this regard. SKF 525A often inhibits these microsomal reactions.

The livers of the eight species examined all activated schradan, the most effective being the rat, the least effective the rabbit. Twelve tissues of the rat were studied and (contrary to previous reports) several besides the liver showed weak activating activity; lung, heart, and testis were effective; small intestine and stomach less so; ovary, pancreas, voluntary muscle, kidney, spleen, and brain were inactive.[72] This is in agreement with the observation by Cheng[24] that the cholinesterase of the hepatectomized rat was partially inhibited by the administration of schradan, even though the absence of the liver gave a clear protective effect.

An attempt has been made[73] to explain the anomaly that oxidation of schradan requires a reduced pyridine nucleotide. An obvious guess was that peroxide is involved since it is an oxidizing agent which is a common product of the oxidation of DPNH. It was shown that H_2O_2 is indeed produced by the microsomal oxidation of DPNH, and on this and a number of other pieces of evidence, the (highly tentative) scheme (I) was proposed to describe the action of liver + DPN, where X is a system located in the microsomes, and AH_2 is an oxidizable substrate. The various steps have all been

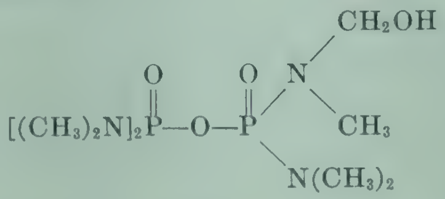


(I)

shown individually *in vitro*, but there is as yet no evidence that this is the scheme that normally operates.

The ethyl analog of schradan is not activated by liver microsomes, nor is Tetram, which contains a diethylamino group.[75] The system appears to be specific for dimethylamides.

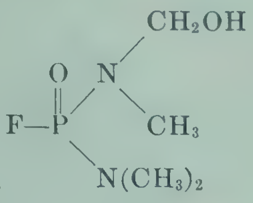
The identity of the active anticholinesterase metabolite of schradan has been the subject of some dispute. However, it is generally agreed that the same compound is produced by activation in mammals, insects, and plants, and by oxidation with permanganate and hypochlorite[20, 21, 22, 45, 78, 84]; the problem, therefore, simplifies to the identification of the chemical product. This matter is discussed in detail in Chapter 2; the active product is considered to be, not the *N*-oxide as the earlier work suggested, but hydroxymethyl schradan (II).



(II)

Dimefox is another phosphoramide: $[(CH_3)_2N]_2P(O)F$. Aldridge and Barnes[7] showed that liver slices converted it to a potent anticholinesterase. A thorough study of this effect was made by Fenwick, *et al.*[37] Livers from male rats were about 4 times as effective as those from females, much like schradan. But of the three species tested, the liver of the rat was the least effective and that of the rabbit was the most effective; the findings are reversed with schradan. The work was made difficult by the excessive instability of the metabolite which is hydrolyzed in a few minutes at 37°C. near pH 7. The activation was inhibited by SKF 525A, 2,4-dinitrophenol, barbiturates, and anaerobiosis. Liver homogenates were inactive, but on addition of magnesium, nicotinamide, and DPN (or TPN), activity was demonstrated. The eight other mammalian tissues studied contained no activating system.

The activation of dimefox by liver slices has been confirmed by Arthur and Casida.[9] These workers also investigated the nature of the potent metabolite and concluded that it was directly analogous to that of schradan. We may therefore tentatively consider it as the hydroxymethyl derivative (III).



(III)

However, Arthur and Casida feel that it may be the *N*-oxide structure. Which structure is accepted depends, at the moment, entirely on whether one considers the schradan metabolite to be the *N*-oxide or the hydroxymethyl derivative; the evidence on this matter is discussed in Chapter 2.

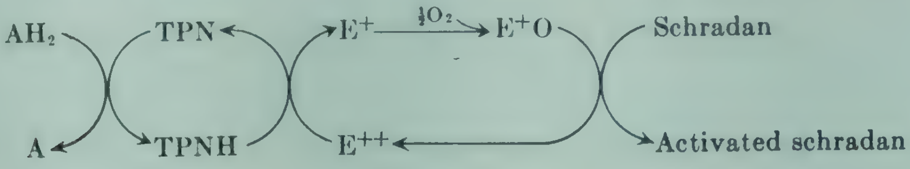
Phosphoramidate Oxidation by Insect Tissues

The activation of schradan by insect tissues was first inferred by Duspiva[35] who in 1951 observed that the cholinesterase of *Pyrrhcoris apteris* was inhibited by schradan *in vivo*, yet was insensitive *in vitro*. In 1953, O'Brien and Spencer[77] showed that several tissues from the five species of insects studied all activated schradan *in vitro*. The five species included three (American cockroach, mealworm larva, and mourning-cloak larva) which were not susceptible to schradan poisoning. The insect system resembled the system of liver homogenate in its requirement for oxygen and in its susceptibility to most inhibitors, except that chloropicrin was a poor inhibitor. The active metabolite was identical with that produced by permanganate oxidation or metabolism by liver.[78]

In 1954, Casida *et al.* showed that the nine tissues studied from the American cockroach were effective activators, the best being that of the gastric cecum.[21]

Fenwick,[36] in 1958, was the first to obtain phosphoramidate activation with a broken cell preparation from insects. He noted that schradan conversion was vigorous with whole fat body of locust, but not with whole gut. The fat body activity varied markedly with age: activity declined sharply in fifth instar insects two days before moulting, and after moulting returned slowly to stabilize at about one-fifth of the fifth-instar level. Homogenized locust fat body was effective in activating schradan, and its activity was improved by Ca^{++} or Mg^{++}, but not by DPN, TPN, or nicotinamide (c.f. liver system, above). The lack of requirement for nicotinamide was attributable to low nucleotidase activity. The homogenate could be fractionated centrifugally, yielding supernatant and a particular fraction, neither of which were effective separately, but which were fully active if combined. The supernatant could be replaced by TPNH but not by DPNH, an interesting difference from the liver system. The failure of TPN to improve the whole homogenate was attributed to a sufficient endogenous level. The requirement for Ca^{++} or Mg^{++} disappeared if TPNH was provided, showing that these ions were needed for the TPN-reducing system of the supernatant.

The system was inhibited by numerous compounds, including an —SH inhibitor (*p*-chloromercuribenzoate), an uncoupling agent (2,4-dinitrophenol), and metal chelaters such as Versene (ethylenediamine tetraacetic acid). Fenwick suggested that a metallo enzyme was involved, since (*a*) Mepacrine inhibited, and it is a metallo-enzyme inhibitor; (*b*) a nonenzymic mixture of ascorbic acid, ferrous iron, and Versene could activate schradan. This proposal would suggest that the nucleotide functions by keeping the metal in the reduced state. Fenwick suggested that the over-all scheme (IV) might be as shown; E^{++} is the oxidized enzyme and E^{+} is the reduced form. (This is a somewhat abbreviated form of Fenwick's formulation.)



(IV)

The activation of dimefox by whole cockroach guts has been demonstrated.[37] Homogenization destroyed the activity, and DPN, Mg^{++}, and nicotinamide did not restore it. Fenwick's locust fat body system also activated dimefox.[36]

Phosphorothionate Oxidation by Mammalian Tissues

Diggle and Gage[31] pointed out in 1951 that although parathion was a poor inhibitor of cholinesterase *in vitro*,* it was a potent inhibitor *in vivo*. Furthermore, the dose of parathion required to produce 50% inhibition, *in vivo*, of rat brain cholinesterase was little different from the dose of *S*-ethyl parathion isomer, even though the latter is a vastly more potent inhibitor *in vitro*. They also observed that whereas the *S*-ethyl isomer produced rapid inhibition of cholinesterase *in vivo*, parathion administration only produced inhibition after a lag period. On this basis, the authors argued that an activation must occur *in vivo*, and proceeded to show that liver slices could activate parathion *in vitro*. This was confirmed by Metcalf and March,[53] who also showed activation by liver slices of methyl parathion and acethion amide.

The capacity of liver preparations to activate parathion is completely lost on homogenization, and this phenomenon hindered further studies on the activating system. Davison[29] found that the activating capacity of rat liver homogenates could be restored by adding magnesium and DPN (diphosphopyridine nucleotide) along with nicotinamide, precisely as he had found for schradan activation. The activity of the fortified homogenate was due almost exclusively to the microsomal and soluble fractions: these fractions had some activity by themselves, but both were needed to obtain strong activation. The sensitivity of the parathion-activating system to

* This important observation denied earlier reports to the contrary, as discussed in Chapter 2. Only preparations which have been purified show low anticholinesterase activity.

various inhibitors was substantially the same as that for schradan activation, described above.

The activation of Guthion by liver slices was first shown by DuBois *et al.*[34] More extensive work by Murphy and DuBois[68] with liver homogenates was complicated by the enzymic degradation of the anticholinesterase metabolite by liver phosphatases; the problem was largely avoided by using low tissue concentration. These authors' findings differed from those of Davison in the following ways. (*a*) Magnesium was not required; but Davison's reported data for cofactor requirements was for schradan activation, and the conclusion was perhaps incorrectly extrapolated to cover parathion activation. (*b*) Their best microsome preparation contained almost all the activity and was not much improved by adding the supernatant fraction. (*c*) Male rat livers yielded microsome preparations four times more active than those of female (Davison found the reverse with parathion). The sex difference was less marked in mice and guinea pigs

Murphy and DuBois' activating system resembled Davison's in being more active with DPN than with TPN, in being inhibited by SKF 525A, and having optimum activity near pH 7. Some interesting new observations made by these authors were that the system was inhibited by $8 \times 10^{-5}\ M$ cytochrome c (in contrast to the schradan-activating system[73]) and by prior incubation with pyridoxal phosphate, but not by riboflavin or pyridoxine, and very little by arsenite.

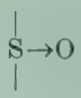
Later work on phosphorothionate activation by O'Brien[75] showed that for parathion activation DPNH was more effective than DPN in a washed microsome preparation (presumably similar to that of Murphy and DuBois), that the same was true of TPNH and TPN, and that the latter two were better than DPNH and DPN, respectively. Using a system containing washed microsomes, DPNH, magnesium, and nicotinamide, activation of the following phosphorothionates was found: parathion, Co-ral, malathion, Guthion, EPN, Diazinon, Potasan, ronnel, and the thiono analog of Tetram. However, such a system did not activate dimethoate, acethion, prothion, methprothion, or ketothion, probably because the microsomes can degrade these compounds hydrolytically.[75, 79] But activation of dimethoate by mouse liver slices has been reported by Santi and De Pietri-Tonelli.[81]

Thus, there are a number of discrepancies between the reports of the three principal groups of workers. At the moment there are too many differences in the conditions of study to justify a critical assessment. It will be necessary for one group (preferably a fourth one!) to examine the discrepancies using one standard system, and parathion, Guthion, and schradan.

The identity of the activated phosphorothionate was considered in the case of parathion. Two possibilities were considered: that the product was paraoxon, or that it was the *S*-ethyl isomer. Myers *et al.*[70] showed in 1952 that of these two possibilities, the former was preferable, since the relative inhibition *in vivo* of true cholinesterase and of aliesterase (judged by tributyrin hydrolysis) was very similar for parathion and paraoxon, but different in the case of the *S*-ethyl isomer of parathion. Similarly, for the compound methyl bis (*p*-nitrophenyl) phosphorothionate, the parent and its P═O analog gave one pattern of *in vivo* results, whereas the *S*-methyl isomer showed a different one.

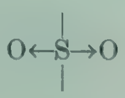
More direct evidence was given by Gage and Payton[41] and Gage.[40] They treated rats with parathion and at death (10 minutes later) removed the livers and extracted them with acetone. The extract was chromatographed on a cellulose powder column and was separated into 3 fractions, all absorbing at 265 mμ. The second fraction contained all the anticholinesterase activity, and was a single component as judged by rechromatography on a similar column and by paper chromatography. This component was identified as paraoxon on the basis of an identity of anticholinesterase activities, ultraviolet spectra, chromatographic behavior on paper (one solvent system), and hydrolysis constants.

Although evidence is lacking in most cases, it may be taken as a general rule (except for compounds containing the C—S—C system) that activation of phosphorothionates is achieved by oxidation to the corresponding phosphate. As for compounds such as Systox and Ekatin which contain the thioether group, C—S—C, no work is available upon the activation *in vitro*. However, information derived from *in vivo* studies (pages 217–232) suggests that in these cases oxidation of the thioether sulfur to the sulf-



$\overset{|}{\underset{|}{S}}\rightarrow O$

(I)



$O\leftarrow\overset{|}{\underset{|}{S}}\rightarrow O$

(VI)

oxide (V) or sulfone (VI) derivative is as important in activation as oxidation of P═S to P═O.

$(C_2H_5O)_2P(S)OCH_2CH_2SC_2H_5$

Systox (thiono form)

$(CH_3O)_2P(S)SCH_2CH_2SC_2H_5$

Ekatin

Murphy and DuBois[69] have studied the way in which activation of the phosphorothionate Guthion varies with age and sex. For these experiments, whole liver homogenate, fortified with nicotinamide and DPN, was used. In rats up to 30 days old, the activity was similar in males and females, but thereafter it varied as shown in Fig. 4.5. By administering testosterone for six weeks to female rats, the Guthion-activating activity of their livers could be raised to a male-like level; by castrating young male rats the in-

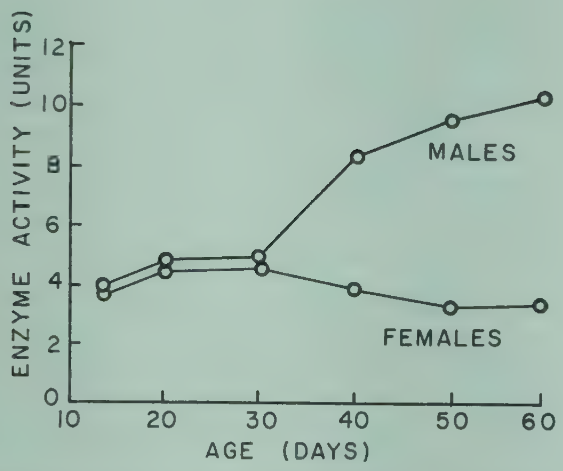


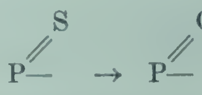
FIG. 4.5. Variation with age of Guthion-oxidizing activity in rat liver. From Murphy and DuBois.[69]

creased activity after 30 days was eliminated; and by administering diethylstilbestrol or progesterone for a month to adult males, their activity was reduced to a female-like level. Age and sex are thus of major importance in determining the extent of activation.

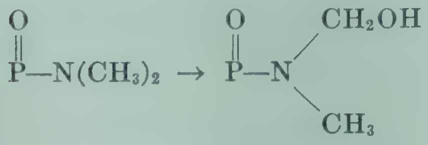
The results for Guthion contrast with those for parathion,[29] which is activated ten times better by female livers than by male livers. In the case of parathion, the toxicity corresponds to the activating ability: females are twice as susceptible as males.[33] For Guthion there is no such correspondence, for the females are again twice as susceptible,[34] in spite of their lesser activating ability. This is not cause for surprise; many other factors, such as degrading ability, undoubtedly contribute to the over-all toxicity.

The level of the Guthion-activating enzyme in regenerating liver is low —i.e., the liver tissue grows back more rapidly than the enzyme is synthesized. Administration of a single dose of the carcinogen, 3-methylcholanthrene, to adult females or weanling males increased the liver activity two or threefold, but the compound had little effect with adult males. It seems as if the adult male has a liver with maximal activity (this was shown experimentally not to be caused by a soluble activating factor in livers of the adult male). The stimulating effect of 3-methylcholanthrene was completely blocked by prior treatment with ethionine. Both the stimulation and its blockade duplicated the action of these agents upon another microsomal system which demethylates 4-dimethylaminoazobenzene.

Is the same system responsible for the activation by liver of phosphorothionates and phosphoramidates? Although the biochemical outcome of both events is similar, i.e., a weak anticholinesterase is activated to a strong anticholinesterase, the chemical changes involved are radically different:



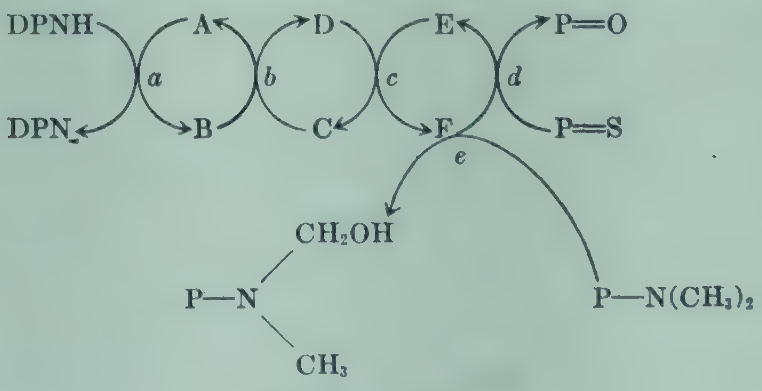
Phosphorothionate activation



Phosphoramidate activation

It therefore seems improbable that a single system is involved. However, the cofactor requirements and behavior towards inhibitors are closely similar for both reactions. But there is a small difference in pH optima, and whereas the ratio of activities of female to male rat livers is about 8 for parathion, it is about 0.5 for schradan.[29]

One must also bear in mind the incredible variety of reactions catalyzed by liver microsomes and reduced nucleotides, e.g., oxidation of barbiturates, dealkylation of some amines, cleavage of aromatic ethers, hydroxylation of phenylalanine, reduction of some ketosteroids, cyclization of squalene, and conversion of tetraethyltin to triethyltin.[19] Many of the reactions are inhibited by the same compounds (particularly by SKF 525A), but it seems impossible to postulate a single enzyme because this would require an extraordinary degree of nonspecificity. The most probable explanation is that there are a series of fairly specific enzymes all "fed" by the same DPNH-utilizing oxidation scheme. SKF 525A, anaerobiosis, and so on may interfere with one of the common steps shown in Scheme VII. Here, A, B, C, D, E, F are cofactors or intermediates; *a*, *b*, *c*, *d*, and *e* are enzymes. A requirement for oxygen and magnesium, and a sensitivity to SKF 525A should be present in one or more of *a*, *b*, and *c*. Systems *d* and *e* would be the stages conferring relative specificity. Such a system is closely analogous to the carbohydrate-oxidizing systems; enzymes such as α-ketoglutaric de-



(VII)

hydrogenase and glyceraldehyde-3-phosphate dehydrogenase are highly specific for substrate, but produce a common product, DPNH, which is oxidized to DPN and water (with concurrent energy gain) by a single system, the flavine-cytochrome chain.

The problem will only be elucidated when microsomes have been fractionated; this requires solubilization first, and all attempts at solubilization have resulted in a loss of activity. The loss could be due to a requirement of the solubilized preparations for terminal electron acceptors other than oxygen, a possibility that has not been investigated at present. Another approach would be the discovery of inhibitors specific to the initial enzymes, which would thus inhibit (say) the schradan-activating but not the parathion-activating capacity of a microsome preparation.

PHOSPHOROTHIONATE OXIDATION BY INSECT TISSUES

Activation by insect tissues was found first by Metcalf and March.[53] They showed that flies poisoned by phosphorothionates had their cholinesterase strongly inhibited, yet the pure phosphorothionates were poor inhibitors of fly-brain cholinesterase. Furthermore, the toxicity of phosphorothionates to insects was not markedly less than their corresponding phosphates.[54] Consequently, they suspected activation by insects, and showed[53] that cockroach gut activated parathion, methyl parathion, malathion, EPN, and acethion amide.

More detailed studies[53] were then focused on the activation of methyl parathion by cockroach gut. The activation was aerobic and heat-sensitive, and was eliminated by homogenization. Activity in phosphate buffer was greater than in veronal buffer, as contrasted to the schradan-activating system described above. Numerous insect tissues accomplished the activation: all parts of the gut, malpighian tubules, fat body, and nerve cord. Fore-gut showed greatest activity; cuticle and muscle were inactive. A variety of inhibitors were effective in blocking activation, particularly sulfhydryl inhibitors (chloropicrin and iodoacetate) and metal-enzyme inhibitors (cyanide and azide). However, inhibitors of carbohydrate oxidizing enzymes (malonate and fluoroacetate) were not potent inhibitors: this is in harmony with their observation that activation *in vitro* was not enhanced by glucose.

Chromatographic data indicated that the products of activation of parathion and methyl parathion by cockroach fore-gut were the respective P=O analogs.

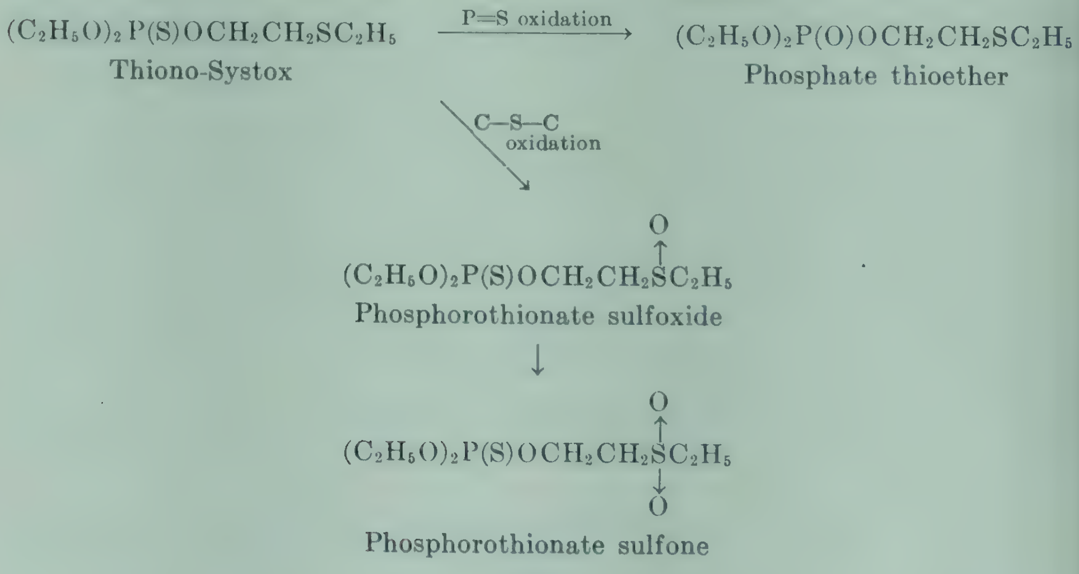
In general, then, the thionophosphate-activating system of insect gut resembles that of mouse liver, except that cyanide may be a poor inhibitor of the liver but a good inhibitor of the gut system. We must say "may be" because the liver was unaffected by 10^{-4} M cyanide whereas the gut was affected by 10^{-3} *M* cyanide, and it is possible that this concentration factor is critical. Furthermore, cyanide concentrations are often uncertain, due to the volatility of HCN.

Kok and Walop[49] studied parathion activation in the American cockroach and found the same tissues were effective as Metcalf and March had reported for methyl parathion, but reported that fat body was by far the most effective tissue. Malathion activation has been shown in all parts of cockroach gut, and in fat body which was the most active tissue.[74] Tissues of the cattle grub and housefly have been shown to activate the important animal-systemic insecticide, Co-ral.[80]

As with liver preparations, homogenization of insect tissues eliminates activating capacity, and in fact may reveal degrading capacity. Addition of DPN, magnesium, and nicotinamide to a homogenate of cockroach gut restores a little of the capacity to activate malathion,[74] in contrast to the ineffectiveness of these cofactors in restoring the schradan-activating capacity of the same preparation.

THIOETHER OXIDATION

The thioether group (C—S—C) is found in compounds of the Systox type. A number of possible oxidative activations can (in principle) occur. For instance, with thiono-Systox one could have the scheme shown below (VIII).



$(C_2H_5O)_2P(S)OCH_2CH_2SC_2H_5 \xrightarrow{P{=}S\ \text{oxidation}} (C_2H_5O)_2P(O)OCH_2CH_2SC_2H_5$

Thiono-Systox — Phosphate thioether

C—S—C oxidation

$(C_2H_5O)_2P(S)OCH_2CH_2\overset{\overset{O}{\uparrow}}{S}C_2H_5$

Phosphorothionate sulfoxide

↓

$(C_2H_5O)_2P(S)OCH_2CH_2\underset{\underset{O}{\downarrow}}{\overset{\overset{O}{\uparrow}}{S}}C_2H_5$

Phosphorothionate sulfone

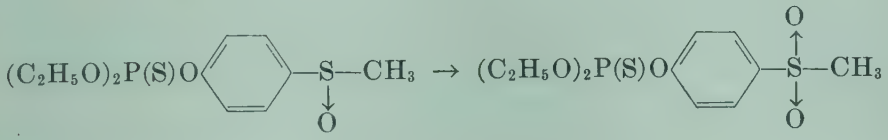
VIII

Furthermore, one may have the sulfoxide and sulfone derivatives of the phosphate. Similarly the thiolo isomer of Systox, containing the P(O)S— group, may have its thioether sulfur oxidized to the sulfoxide or sulfone.

Unfortunately, the *in vitro* studies on these oxidations have not used methods which can distinguish the thionophosphate sulfone from the phosphate sulfoxide or sulfone, nor the thiolophosphate sulfoxide from its sulfone. Consequently, definitive data are lacking. However, March, *et al.*[51] showed that mouse liver oxidizes thiono-Systox by thioether oxidation, so that no phosphate thioether was found. The major metabolite was the phosphorothionate sulfone. Extensive thioether oxidation of thiolo-Systox by liver was also shown. Muscle, kidney, and brain were inactive. In the American cockroach, a substantially similar metabolic pattern was found, but in this case gut, muscle, and nerve, all had some activity.

It seems then that as far as Systox is concerned, activation by phosphorothionate oxidation is small, and thioether oxidation is the principal activation route *in vitro*.

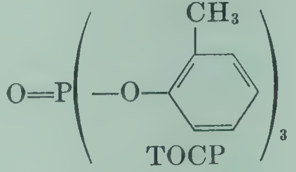
Benjamini *et al.*[17] showed that Bayer 25141 was oxidized by roach gut *in vitro* to a sulfone:



Bayer 25141

Triphenyl Phosphate Activation by Liver

Triorthocresyl phosphate (also known as TOCP, TCP, triorthotolyl phosphate, and TOTP) has been considered for many years to be an anticholinesterase "in its own right."



TOCP

In 1954, Aldridge showed that carefully purified TOCP was a very poor anticholinesterase *in vitro*, but was converted to a potent one on injection into the rabbit or chicken, or by incubation with rat-liver slices. Such an activation did not occur with the meta and para isomers of TOCP.[6] Myers *et al.* suggest, from analogy with the metabolism of *m*-dichlorbenzene and *o*-tolylurea, that the potent metabolite is a *p*-hydroxylated derivative, or a metabolite of such a derivative.[71]

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CHAPTER 5

Effects on Isolated Whole Tissues

Previous chapters have dealt with the reaction of organophosphates either with simple enzymes or with such disorganized systems as whole homogenates. In this chapter we shall consider some effects upon a few organized tissues, principally muscle and nerve, which can be studied in isolation. This isolation enables one to exclude, in most cases, such factors as degradation, activation, or excretion. It should help us to assess the contribution of direct tissue actions in the whole animal, and to evaluate the role of modifying enzymes in that more complex situation.

Ion Permeability

A number of papers by Greig and her co-workers have supported the thesis that acetylcholine has an important influence upon the permeability to ions of numerous tissues, and that in these tissues anticholinesterases act by disrupting the acetylcholine balance and so producing ionic derangement. Thus, eserine at 10^{-5} M reduced the permeability of erythrocytes to sodium and potassium.[29, 31, 32] Acetylcholine had the same effect.[28, 48, 57] Eserine also reduced the fragility (i.e. susceptibility to hemolysis) of erythrocytes and decreased the permeability of the brain to acid fuchsin[30] and barbital.[33] Other esters, such as triacetin and ethyl propionate, reduced permeability of erythrocytes; the activity of all esters was in the same order as their hydrolyzability by erythrocyte cholinesterase.[31]

Similar conclusions followed from a study by Van der Kloot[83] of the active sodium extrusion from isolated frog muscle. Eserine (10^{-3} M) and a crude TEPP preparation (5×10^{-4} M) inhibited sodium extrusion, and in the case of eserine there was a parallelism between the level of intracellular cholinesterase and the sodium loss at various inhibitor concentrations (intracellular cholinesterase was defined as that enzyme in whole muscle which was unavailable to ionized inhibitors but available to ethyl chloroacetate: a very inadequate substrate, since its specificity has never been shown). Van der Kloot considered that a connection existed between cholinesterase inhibition and sodium loss. However, since the glycolytic inhibitor, iodoacetic acid, also diminished sodium loss, it seems probable that the effects of these high anticholinesterase levels are attributable, as in the case of hen brain (see below), to inhibition of energy-yielding metabolism, particularly glycolysis, rather than of cholinesterase.

Again, high concentrations of anticholinesterases were shown by Kirschner[55] to affect the permeability of isolated frog skin to sodium ions. TEPP at 6×10^{-3} M caused total and irreversible inhibition of sodium flux; eserine caused reversible inhibition (no data given). These effects followed application to the inside of the skin; application to the outside produced only small effects. The acetylcholine antagonist atropine (3×10^{-3} M), which was only applied to the outside, induced a 2.5-fold increase in sodium flux. The high concentrations which were needed suggest, as in the case of erythrocytes, that these effects may not be due to cholinesterase inhibition.

Other workers have shown that there is a marked difference between the amounts of organophosphate needed to inhibit cholinesterase completely and the amount to effect ion permeability. Thus Taylor *et al.*[78] found that about 10^{-3} M DFP was needed to affect potassium diffusion in erythrocytes, yet 10^{-5} M completely inhibited cholinesterase, a disparity of one hundredfold. With eserine the disparity was tenfold. For active transport of sodium, a comparable disparity was found with paraoxon; but the slow passive diffusion of sodium was somewhat more sensitive.[79] Working with schradan-poisoned patients, Goodman *et al.*[27] showed that drastic (80%) inhibition of the cholinesterase of erythrocytes had little effect upon their potassium permeability.

Strickland *et al.*[76, 77] carried out very careful studies on the effect of anticholinesterases upon potassium diffusion in cerebral slices of hen brain. At high concentrations, DFP and some carbamate inhibitors did indeed greatly increase the potassium permeability of the slices—but only at levels far in excess of those required to inhibit cholinesterase: there was a 200-fold difference between the maximum concentration which showed no effect on potassium permeability and the minimum concentration needed to inhibit cholinesterase 100%. They found that the concentrations of inhibitor needed to affect potassium diffusion also decreased oxygen uptake and oxidative phosphorylation, and increased carbon dioxide output. Therefore it seems that at least as far as DFP and brain slices are concerned, the potassium permeability effect is caused by some disruption of the respiratory process, and is not related to inhibition of cholinesterase.

Studies along quite different lines have thrown light on another general role of acetylcholine: Hokin and Hokin[41-43, 46] have examined the striking effects that very low concentrations of acetylcholine (e.g., 10^{-6} M) have upon the products of secretory tissues, e.g., mucin and amylase from salivary gland, adrenaline from adrenal medulla, and pancreatic juice from the pancreas. In all these cases, acetylcholine greatly increased secretion of the isolated or sliced gland. These effects were accompanied by a curious biochemical change: the rate of turnover of phosphate in certain phospholipides was greatly stimulated, but the nonphosphate part of these molecules was unaffected. Similar biochemical effects were seen in brain slices and microsomes.[44] Atropine was observed in several cases to abolish the acetylcholine effect. In several tissues such as liver, kidney, and heart, whose function is not primarily secretory, no effect of acetylcholine upon phospholipid turnover was found.

This stimulation of secretion is probably not connected with ion movement. In brain slices, combinations of acetylcholine and eserine in low concentrations had marked effects upon phosphorus turnover with no effect upon ion permeability.[40] It may seem surprising that these two penetration phenomena are different. The Hokins believe that the secretion effect is a stimulation of active transport of hydrophilic materials which are rendered liposoluble by transient combination with phospholipids, and whose capacity to cross lipid membranes is thereby enhanced. There would, therefore, be no reason to expect such an effect to influence ion permeability.[40] It may be, however, that at much higher acetylcholine concentrations the above system breaks down and the ion-permeability is affected. Indeed, Hokin *et al.* noted that high concentrations of acetylcholine (e.g., 10^{-3} M) had an effect upon phospholipids that was quite different from that of low concentrations: instead of a selectively stimulated phosphorus uptake, a nonspecific depression of uptake was found.[46]

We may expect that these secretion-stimulating effects of acetylcholine will be mimicked by low concentrations of anticholinesterases *in vivo*, or *in vitro* where an acetylcholine source is present. This has been observed for TEPP at 10^{-5} M with slices of various salivary glands[45] and it is implied by the fact that most of the effects found with acetylcholine were maximal only if eserine was added. Copious salivary excretion is a feature of organophosphate poisoning. We cannot say how much of this is due to an effect upon autonomic innervation and how much to a direct stimulation along the lines just indicated.

Muscle Contraction

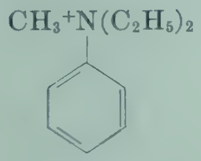
It is well-known that acetylcholine can cause contraction in isolated muscles of all kinds; in fact, this phenomenon is used to bioassay acetylcholine, using such varied muscles as the rectus abdominis of the frog, the heart of the clam, and the ileum of the rabbit. In such assays, an anticholinesterase (usually eserine) is used to avoid destruction of the acetylcholine by tissue cholinesterases. This use of an anticholinesterase is called "sensitization," since it greatly increases the responsiveness of the preparation to acetylcholine.

The simplest theory of the mode of action of organophosphates upon a muscle *in situ* or upon an isolated muscle in the presence of added acetylcholine would be as follows: the organophosphate inhibits the cholinesterase at the motor end-plate, thus permitting the local concentration of acetylcholine to rise and either overstimulate the end-plate or depolarize it completely.

In 1955, Cohen and Posthumus[10] first suggested that in the frog rectus preparation, sensitization by DFP, sarin, and eserine is not solely due to the inhibition of cholinesterase or other esterases. Their reasons were that (*a*) these three anticholinesterases sensitized the muscle to butyrylcholine and succinylcholine, although there are no esterases in the muscle which hydrolyze these esters; and also sensitized it to such nonesters as decamethonium, Tensilon, and choline.

$$(CH_3)_3\overset{+}{N}{-}(CH_2)_{10}{-}\overset{+}{N}(CH_3)_3$$

Decamethonium



Tensilon

(*b*) The DFP-treated sensitized muscle could be washed repeatedly and then the sensitization was reduced or eliminated; but the cholinesterase remained totally inhibited.

The explanation suggested was as follows: perhaps the receptor sites for acetylcholine (i.e., those sites whose stimulation by acetylcholine induces muscle contraction) resemble cholinesterase in having an esteratic (or B) site and anionic (or A) site; and perhaps the requirement for receptor stimulation is that both its A and B sites are occupied. If, then, the anticholinesterase combined with the B site of the receptor, subsequently added acetylcholine would only have to attach to the A sites. In order to explain the findings, the attachment of anticholinesterases to the B sites of the receptor would have to be reversible; that to the B site of the cholinesterase is of course relatively irreversible.

Van der Meer and Meeter[84] obtained evidence of a similar situation in the isolated rat nerve-diaphragm preparation: DFP decreased the critical nerve stimulation frequency for contraction, and even when cholinesterase was eliminated, further DFP gave a further decrease. Similarly, with zero cholinesterase further DFP increased the sensitivity to acetylcholine. Subsequently Cohen and Posthumus[11] showed for this preparation effects identical with those that they had described for frog muscle, i.e., sensitization by DFP to butyrylcholine, Tensilon, and so on. Barnes and Duff[1a] had noted in 1953 that when such a preparation was treated with paraoxon, the power to sustain a tetanus (i.e., a sustained contraction following extreme stimulation of the nerve) was abolished, but that on removal of the inhibitor, recovery of tetanus capacity occurred much more rapidly than recovery of cholinesterase.

The results described so far seem to indicate conclusively that DFP and paraoxon have actions on muscles other than those due to cholinesterase inhibition. However, Fleisher *et al.*,[23] working with sarin on the frog rectus muscle, report findings that imply that the desensitization which Cohen and Posthumus achieved by washing was in fact due to fatigue of the muscle. In order to achieve desensitization without washing, Fleisher *et al.* used the cholinesterase reactivator 2-PAM (page 102) on the sarin-treated muscle. The 2-PAM induced desensitization with concurrent recovery of cholinesterase. The quantitative agreement between per cent decrease in sensitization and per cent cholinesterase recovery was particularly good if the cholinesterase was assayed on whole intact muscle. Desensitization along with cholinesterase recovery could also be achieved by washing with acetylcholine solution, and once more the correlation between enzyme recovery and loss in sensitization was better if the cholinesterase of the intact muscle (rather than homogenates) was studied: under these conditions the recovery of cholinesterase was 24% and of sensitization, 61%. This work of Fleisher *et al.* therefore casts a little doubt upon part of the findings of Cohen and Posthumus. Yet the latters' observation that DFP sensitizes to Tensilon, etc. must imply an action other than that of cholinesterase inhibition.

Further doubt as to whether the effects of organophosphates were exclusively due to the inhibition of cholinesterase was raised by the work of McNamara *et al.*[59, 60]; they found that in cats poisoned with DFP or TEPP, the cholinesterase of the gastrocnemius muscle was irreversibly inhibited, yet the decrease of tension in the directly stimulated denervated muscle or in the muscle stimulated at its motor nerve was reversible. With TEPP, conductivity of the sciatic nerve and the response of the muscle to stimulation via the nerve could be abolished but would recover in a few hours, even though cholinesterase did not recover. The cholinesterase of nerve was assayed manometrically, of muscle histochemically, and the results were in agreement. They concluded that cholinesterase inhibition could not account for these reversible effects. Later, Groblewski *et al.*[34] found that DFP and sarin caused a direct contraction of the denervated gastrocnemius muscle of the cat; but this effect was quite unrelated to anticholinesterase action, since several phosphates which are not cholinesterase inhibitors showed precisely the same action, e.g., Na_3PO_4, sodium diisopropyl phosphate, etc. They also showed that it was not a simple pH effect, and attributed it to the P(O)O-grouping.

The concept of a dual action of DFP on the rat diaphragm, stimulated via the phrenic nerve, and on rabbit aortic strip (a smooth muscle preparation), stimulated by acetylcholine, was also advanced by Naess.[62] He found that large concentrations of DFP (greater than 10^{-3} *M*) depressed the sensitivity of these preparations reversibly, and he suggested that there was a depressive effect on the acetylcholine receptor. Such experiments using gigantic inhibitor concentrations are however of little importance in evaluating *in vivo* effects.

Erdmann and Heye[19] showed direct effects of very small concentrations of Systox, parathion, and paraoxon upon isolated rabbit intestine. At about 10^{-8} M, the compounds increased the pendular motility and tone of rabbit intestine, and paraoxon also induced peristalsis. These stimulating effects were probably due to the inhibition of cholinesterase, as judged by the antagonism of agents such as atropine and 2-PAM and the failure of washing to restore the preparation. At higher levels of inhibitor (10^{-5} M) a paralysis was seen, probably due to a direct action unrelated to cholinesterase inhibition, as judged by the ineffectiveness of 2-PAM and the reversibility of the effect after washing the preparation.

In summarizing these muscle effects, it seems that there is good reason to doubt whether effects upon muscle can be explained entirely on the basis of cholinesterase inhibition. The alternative theories require some form of direct reaction between organophosphate and the acetylcholine receptor. It seems that such direct combinations are most liable to occur at relatively high concentrations of organophosphate. Nevertheless it must be considered as a distinct possibility that some of the consequences of organophosphate poisoning in mammals are caused by such a combination.

Nerve Transmission

The electrophysiology of mammalian and insect nerves has been studied in some detail, and data upon the effects of organophosphates upon them are obviously important for our subject. A good deal of data has been obtained from the squid, lobster, and frog, primarily on grounds of convenience of such preparations. A reminder about the taxonomic relationships between these various animals is found in the tabulation.

Phylum	Class	Example
Mollusca	Cephalopoda	Squid
Arthropoda	Crustacea	Lobster and crayfish
	Insecta	Insects
Chordata	Amphibia	Frog
Subphylum Vertebrata	Mammalia	Rat, cat, and dog

For the purpose of this book, perhaps only data upon insects and mammals should be given. But information upon the effects of organophosphates upon nerves is rather limited, and since many of the effects upon one group also hold for the others, we shall include data for these other classes.

A general introduction to the nervous system is given in Chapter 1. In the results below, the terms "nerve" and "axon" are used a good deal. It may help to recall that a nerve (which usually implies a nerve trunk) is a bundle of axons, and a ganglion contains synapses. The transmission in nerves is entirely axonic, and in ganglia it is axonic and synaptic. In the squid a single axon, the giant axon, has often been studied as it provides a simpler picture. The cell membrane or axolemma of the nerve axon is polarized, i.e., there is a potential difference (the membrane potential) between the inside and outside. During passage of an impulse, this potential decreases to zero, overshoots, and then returns to its resting condition; these events are called the *action potential*. If a nerve is injured, the membrane potential is lowered at the point of injury. The potential difference between intact and injured portions is the *demarcation potential*.

As the observations to be described are elaborate and in some cases under dispute, we shall begin by two brief paragraphs giving what seem to be the most important conclusions. This may help by giving some shape to the subsequent detailed discussion.

Axonic transmission in all animals is insensitive to acetylcholine, and is sensitive only to large concentrations (e.g., $10^{-3}\ M$) of organophosphate; in insects even larger concentrations are needed. Synaptic transmission is probably sensitive to low concentrations (e.g., $10^{-6}\ M$) of organophosphate in all animals. In chordates it is also sensitive to acetylcholine, in Crustacea and cephalopods it is far less so, and in insects it is quite insensitive to acetylcholine. Neuromuscular transmission in mammals is extremely sensitive to organophosphates and acetylcholine; in insects it is insensitive to these agents.

In intact insect nerve preparations, then, anticholinesterases are effective only at synapses. Acetylcholine is totally ineffective anywhere; this insensitivity is attributable to an ion-impermeable sheath which protects the whole nervous system from ions, and to the fact that neuromuscular transmission is not cholinergic. When the sheath is removed, the ganglion is found to be somewhat sensitive to acetylcholine and other ionized drugs.

ANIMALS OTHER THAN INSECTS

a. Axonic Transmission

Nachmansohn's group [7, 9, 35] studied the effects of high concentrations of DFP on the bull frog sciatic nerve, squid axon, lobster nerve cord, and cat superior cervical sympathetic nerve. The action potential was slowly abolished, at a rate depending upon DFP concentration, but could be restored by washing the preparation. After this restoration, the cholinesterase was about 20% of normal. At the time of the abolition of the potential, the concentration of DFP inside the squid axon was only 0.6% of that outside, showing that penetration was very poor (with eserine, by contrast, the internal level was comparable with the external[20]). With long exposure of nerves to DFP (e.g., 20 minutes for the cat, and 120 minutes for the bullfrog sciatic nerve) the cord was irreversibly affected: its reactivity could not be restored by washing. By contrast, the carbamate eserine did not give irreversibly inhibited nerve no matter how long the exposure. These workers also showed[8] that DFP reversibly blocked the action potential of sensory nerves, e.g., optic and superficial ophthalmic nerves of the skate, and of nerve normally considered to be adrenergic, as in the splanchnic nerve of the bullfrog.

Rothenberg[72] showed the effects of DFP and eserine upon ion movement in squid axon: $2 \times 10^{-2}\ M$ DFP or eserine significantly increased the permeability of the axon to sodium and potassium, e.g., almost doubling the permeability to sodium.

As a result of these and related studies, these workers (generally identified as the Nachmansohn school) are emphatically of the opinion that the block in nerve conduction by DFP is due to the inhibition of cholinesterase. Other workers are equally emphatic in denying this. Some of their objections are as follows.

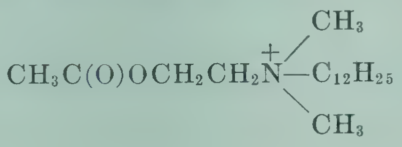
(*1*) Several workers, e.g., from Gilman's and Gerard's group, have found that it is possible to apply DFP to nerve in concentrations, (e.g., $10^{-3}\ M$), such that cholinesterase was almost totally inhibited, yet conduction was still unimpaired.[4, 15, 25] The result has been an argument about techniques; Nachmansohn's group thinks that the manometric procedure used by other workers is insufficiently accurate for the assay of the small levels of cholinesterase (but Boyarsky *et al.*[4] subsequently found no residual cholinesterase using the sensitive acetylcholine disappearance technique); and furthermore that there is often an excess of DFP in DFP-treated nerve which is not in contact with cholinesterase. On homogenization, it is brought into contact and produces an artefactually high inhibition. By correcting for these factors, Nachmansohn's group finds that conduction occurs only if cholinesterase is present.[7, 9, 35] But Crescitelli *et al.*,[15] Boyarsky *et al.*,[4] and Gilman[26] also have experimental evidence that no excess of DFP is usually present.

(*2*) Inhibition of cholinesterase by DFP *in vitro* is irreversible; therefore, only the irreversible component of DFP action can be attributed to cholinesterase inhibition.[80] Bullock *et al.*[9] reported in 1946 that cholinesterase inhibition by DFP *in vitro* underwent a transient reversible phase, passing later to an irreversible type. This appeared to parallel the *in vivo* observations of a transiently reversible blockade. Later, however, Aldridge's criticism of the technique involved in the *in vitro* study was accepted[89] and we may conclude that there is, *in vitro*, no easily reversible DFP-inhibition.

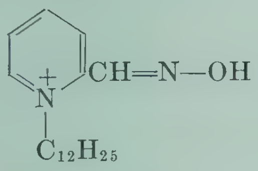
(*3*) At relatively low concentrations, DFP (and eserine and procaine) blocked conduction in frog sciatic nerve without affecting the demarcation potential.[80] Therefore, the conduction block was not due to the depolarization effect which one would expect if acetylcholine normally worked by transiently depolarizing the axon, and if DFP acted by flooding the system with acetylcholine. Instead, Toman *et al.*[80] suggest that DFP acts by decreasing the sensitivity of the nerve by some effect other than inhibition of cholinesterase.

(*4*) High levels of acetylcholine can be added to an axon preparation without affecting it. This anomaly has been satisfactorily explained by Nachmansohn[61] as being due to an ion barrier protecting the axon: thus, the quaternary carbamate prostigmine does not affect conduction, the tertiary carbamate eserine does (cf. page 24). Neuromuscular junctions and the ganglia are not so protected, and here ionized compounds such as acetylcholine have a disruptive effect.

Dettbarn *et al.*[16] have reported that if quaternary nitrogen compounds are modified to give them added liposolubility, they can depolarize and, thus, block conduction in frog axon. The compounds used were:



"Noracetylcholine 12"



Pyridine-2-aldoxime dodecaiodide
(2-PAD)

Similarly, the independent workers Walsh and Deal[85] recently showed that cetyltrimethylammonium bromide blocked conduction in frog axon.

$$(CH_3)_3\overset{+}{N}C_{16}H_{33}$$

Cetyltrimethylammonium bromide

Consequently the failure of acetylcholine to block axonic conduction can be dismissed as a piece of evidence against the role of acetylcholine in axonic conduction, since the failure is readily explained on the grounds of insufficient penetration.

To decide which camp to belong to is not easy for those of us who are not physiologists—most physiologists seem to have strong views on the matter. If one decides with the Nachmansohn group on this matter, that DFP acts on axons by cholinesterase inhibition, this probably implies an acceptance of their view (outlined in Chapter 1) that acetylcholine has a generalized role in conduction, i.e., is vital in axonic as well as synaptic conduction.

The tentative view of the author is as follows. Nerve conduction is associated with ion flux; on this there is no disagreement. As far as certain brain slices and nonconducting tissues are concerned, it is very clear that organophosphates affect ion flux, as we have seen in the preceding section; but it is also clear that this is a high-concentration phenomenon and may therefore have nothing to do with cholinesterase inhibition. Consequently, it is not surprising that nerve phenomena also are affected in one way at high concentrations of organophosphate, and in another way at low concentrations. Furthermore, the ready reversibility of the DFP block finds no parallel *in vitro* anticholinesterase effects. Finally, the failure of DFP to depolarize the axon is difficult to explain on Nachmansohn's views. However, the author is fairly convinced by the demonstration that careful techniques reveal a little cholinesterase so long as conduction is maintained; anticholinesterase activity is the most striking feature of organophosphates. Nachmansohn's opponents have produced no alternative biochemical or physiological lesion. It is certainly possible that in order to allow acetylcholine to accumulate enough to interfere with ion flux, one has to inhibit cholinesterase by, say, 99.9% and this will certainly need very high inhibitor concentrations. The conclusion is that Nachmansohn's views should be accepted as a tentative working hypothesis, but that it must be realized that there are a number of phenomena which it does not explain. Fortunately, axonic effects need not be invoked to explain *in vivo* phenomena—appropriate concentrations are probably never achieved.

From what has been said, it is clear that most ionized materials fail to penetrate the squid axon or the nerve of the other animals studied. In nerves there are other protective barriers beside that which closely invests the axon. In the frog, for instance, a part of the perineurium (a double sheath around the nerve bundle) is responsible for retarding diffusion of potassium ions and ionized drugs.[14, 21, 22, 74] A similar sheath has been shown in the somatic nerves of several mammals.[56] However, these barriers do not provide complete protection against ions (as does, for instance, the locust perineurium) and merely delay the effects of externally applied agents.[39] Thus, all axonic preparations, whether isolated as in the squid axon experiments or in bundles as in most other cases, are sensitive to potassium.[39]

b. Synaptic Transmission

Fortunately, there is relatively little controversy about the way agents affect the peripheral synapse; there is general agreement that acetylcholine is the synaptic mediator. The disagreement is about the role of acetylcholine in axonic transmission. For technical reasons, synaptic studies have usually been made only on those synapses gathered into easily accessible ganglia.

In two excellent studies, Koelle's group[47, 54] studied the effects of DFP upon the superior cervical ganglia of rats and cats. In the cats, DFP was injected and the ganglion studied *in situ*. In rats, the ganglion was removed and immersed in the DFP. In both cases it was found that small concentrations of DFP (e.g., 10^{-6} M in the rat) potentiated transmission and partially inhibited acetylcholinesterase. High concentrations (e.g., 10^{-5} M in the rat) depressed transmission and almost eliminated acetylcholinesterase. The authors felt that the potentiating effect was attributable to cholinesterase inhibition, but were inclined to doubt whether this could also account for the depression. However, it seems to the writer that both effects could be caused by cholinesterase inhibition: partial inhibition (low DFP levels) would give small accumulations of acetylcholine, which are well-known to be stimulatory; severe inhibition would cause large accumulations of acetylcholine, which are well-known to be depressive.

Prosser[67] has shown that the synapses of the sixth abdominal ganglion of the crayfish have a low sensitivity to acetylcholine by comparison with (say) the mammalian ganglion; only at a concentration of 6×10^{-3} M was stimulation, followed by block, observed. This preparation is, however, sensitive to high potassium concentrations as are mammalian nerve preparations.[66] Similarly, in the synapses of the stellate ganglion of the squid, Bryant[5] has shown that numerous ionized drugs have no effect except at high concentrations, e.g., maximum ineffective concentrations were: acetylcholine, 6×10^{-3} M and eserine, 10^{-3} M. Yet changes in the concentrations of magnesium and calcium salts produced effects after a delay period of about 30 minutes. Bullock[6] showed that this ganglion is rather insensitive to DFP: 5×10^{-3} M produced a block only after 7 minutes, and blockade was easily reversed. The sensitivity of the synapse to DFP was, in fact, similar to that of the axon. It seems, then, that crustacean ganglia are better protected against DFP and large ions than are mammalian ganglia. However, it is possible that other organophosphates would be far more effective, as is true for insects (see below).

c. *Neuromuscular Transmission*

The neuromuscular junction is very sensitive to organophosphates; Holmes and Robins[49] showed that the blocking effects of TEPP, DFP, and sarin (e.g., 10^{-6} M) upon various mammalian neuromuscular preparations were reversed by the cholinesterase reactivator, 2-PAM, suggesting that all of the blocking effect was due to cholinesterase inhibition. Douglas and Paton[17] studied TEPP in the cat: small doses (0.5 mg./kg.) sensitized the muscle end-plate to potentiation caused by repetitive stimulation; larger doses (e.g., 2 mg./kg.) rapidly depolarized the end-plate. They felt that the effect was exclusively due to excess acetylcholine, produced either locally or remotely as a consequence of cholinesterase inhibition. Yet the effect was surprisingly reversible: recovery within an hour was usual. Although these two studies support the hypothesis that cholinesterase inhibition sufficiently explains the effects, they are by no means conclusive.

Barnes and Duff[1a] found, in the isolated rat phrenic nerve diaphragm preparation, that low concentrations (e.g., 4×10^{-7} M) of organophosphates (TEPP, paraoxon, and DFP) had two separable effects: (*a*) Tetanus (the sustained contraction resulting from rapid stimulation) was abolished. This effect was reversed by washing, although the inhibition of the true cholinesterase was not so reversed. (*b*) Contraction with a single stimulus was transiently potentiated by paraoxon. The effect disappeared in 10 minutes in spite of the continuing presence of the paraoxon. Even 4×10^{-4} M paraoxon did not block this response. However, with high levels (e.g., 10^{-4} M) of DFP, Barstad,[2] and Berry and Evans[3] were able to show that single-stimulus response in the phrenic nerve diaphragm preparation could be blocked; and that this block could be reversed readily by washing, although cholinesterase did not recover in this washing.

As a corollary to these findings, McNamara *et al.*,[59] working with cats injected with DFP or TEPP, observed a decrease in the tension developed in muscle stimulated via its motor nerve; in 6 hours the single-stimulus response and (to a lesser extent) the tetanic response were very substantially recovered. Yet cholinesterase, measured both histochemically and manometrically, remained totally inhibited.

Neuromuscular transmission, therefore, seems to be blocked by high levels of organophosphate by some mechanism unrelated to cholinesterase inhibition.

It is well known that the neuromuscular junction of vertebrates is sensitive to acetylcholine. In lower phyla the evidence is inadequate.[68]

INSECTS

a. Axonic Transmission

There is an astonishing shortage of data on the effects of organophosphates upon axonic transmission of insects. What there is suggests that, as in mammals, block can only be induced by enormous concentrations. Thus, $10^{-1.2}$ M DFP was needed to block axonic conduction in the American cockroach, and neutralized "HETP," (a preparation containing TEPP), had no effect at $10^{2.5}$ M. The DFP block was readily reversed by washing.[69] In cockroaches, prostrate from a high dose of 5 μg. of TEPP per insect, axonic transmission in the fifth leg nerve, cercal nerve, and ascending fibers of the abdominal cord was completely unaffected; at this dose synaptic transmission in the thoracic ganglia was blocked (E. H. Colhoun, private communication).

b. Synaptic Transmission

The most favored insect preparation (popularized by K. D. Roeder) has been the sixth abdominal ganglion of the American cockroach. This ganglion contains synapses between the sensory nerves coming from the cerci and "giant fibers" (axons of large diameter) which ascend the nerve cord. In 1947, Roeder *et al.*[71] showed that DFP (about $10^{-3}\ M$) had striking effects on the ganglion, causing marked facilitation, i.e., a small stimulus of the cerci causes violent trans-synaptic activity (also called "after-discharge"). The facilitation was followed by total blockage, and these two phenomena alternated in some cases. Transmission in the giant fiber axon itself was quite unaffected. These observations were extended, later, to TEPP,[69] which, however, blocked the synapses at a 1000 times smaller concentration ($10^{-6}\ M$ in place of $10^{-3}\ M$ for DFP).

Roeder[69] was puzzled by the fact that although anticholinesterases are supposed to act by increasing synaptic levels of acetylcholine, acetylcholine and related drugs (atropine, scopolamine, and curare) had absolutely no action if applied to the ganglion even at $10^{-2}\ M$. He concluded that acetylcholine was not the synaptic transmitter. At first, he specifically rejected the possibility that permeability was a factor, perhaps because the carbamates, prostigmine and eserine, were equally potent in causing synaptic block in spite of the quaternarized nitrogen of prostigmine (both blocked at about $10^{-4.3}\ M$),* and the effects were not reversible by washing. Other experiments[70] were carried out with tabun, and sarin and its analogs. With these compounds, an elaborate set of responses was found; with increasing concentration of compound, the reactions were: (*a*) normal, (*b*) facilitation alternating with block, (*c*) normal, and (*d*) block.

To explain this extraordinary phenomenon, Roeder and Kennedy[70] proposed, later, that these organophosphates can, besides inhibiting cholinesterase, block acetylcholine receptors at high concentrations. Thus, the strange period of normality is due to the balance of two functionally opposing processes, one leading to an excess of acetylcholine, the other tending to antagonize the effect of acetylcholine. At an even higher concentration, the antagonizing effect "wins," and a block is seen. The two blocking phases above, (*b*) and (*d*), are, therefore, due to depolarization by an excess of acetylcholine and to blockade of acetylcholine receptors, respectively. This explanation shows that Roeder had accepted the fact that acetylcholine was the synaptic mediator in insects as well as in mammals.

How, then, can one explain the ineffectiveness of applied acetylcholine? A possible clue was provided by Hoyle[51] who showed that the locust nervous system was protected from high potassium levels (which would depolarize the mammalian nerve) by a sheath: injection of potassium under this sheath produced the expected block. The sheath probably resists potassium diffusion actively rather than passively, since its protection is poor when its tracheal air supply is removed.

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* That prostigmine should have an effect is very puzzling. In the locust ganglion, Harlow[36] found that $10^{-4}\ M$ prostigmine had little effect.

This observation suggested to two independent groups, in 1956,[63, 81] that perhaps a nonspecific ion barrier was involved in the locust: if it were present in the cockroach also, it would explain the ineffectiveness of acetylcholine upon the cockroach synapse. If present in all insects, it would explain the uncomfortable anomaly that acetylcholine was nontoxic to insects, although toxic to mammals. This anomaly had led some workers to doubt whether the acetylcholine-cholinesterase system was important in insects.[50, 58]

Twarog and Roeder[81, 82] found that in the American cockroach there was indeed a connective tissue sheath around the nervous system. When this sheath was peeled off the sixth abdominal ganglion, a sensitivity to potassium and acetylcholine was revealed; however, the concentration of acetylcholine was still very large by mammalian standards, e.g., 3×10^{-3} *M*. Many other drugs which were ineffective against the normal synapse had actions upon the desheathed ganglion. These drugs were all such that they would be virtually completely ionized under physiological pH conditions.† Harlow[36] showed a comparable state of affairs for the third thoracic ganglion of the locust; it was insensitive to acetylcholine and other choline esters. In general, ionizable compounds, such as eserine and nicotine, were only effective at high concentrations, e.g., 10^{-3} *M*. Paraoxon and TEPP had effects at about 10^{-5} *M*; both potentiation and block were seen in various preparations. Furthermore, TEPP was equally effective when applied from the outside or by injection, whereas the ionized compound, prostigmine, was one hundred times more effective by injection.

Meanwhile, O'Brien[63] showed that exogenous acetylcholine could not penetrate to the cholinesterase of the perfused American cockroach; such un-ionized substrates as phenylacetate, by contrast, could readily penetrate in this preparation. The effect was attributed to an unspecified ion barrier. Since cholinesterase is restricted to the nervous system in the cockroach,[53] the acetylcholine barrier must invest the nervous system.

Further evidence for the existence of an acetylcholine barrier around insect ganglia and nerves come from the histochemical studies of Winton, *et al.*[90] on the American cockroach and from Wigglesworth[86] on the bug *Rhodnius prolixus*. They used acetylthiocholine as a test reagent and found no staining of the inside of intact ganglia or nerves. Where the surface was cut or damaged, staining did occur, but even in these cases it only extended a short way into the nerves, and when ganglia were cut across, the staining was confined to the exposed surface. Presumably then, there is an effective barrier, but even if this is destroyed, the acetylthiocholine penetrates poorly through the internal tissues. This is in accord with the observation above that even on a desheathed ganglion, very high acetylcholine concentrations are needed to block conduction. The histochemical studies do not constitute rigid proof that acetylthiocholine fails to penetrate: one or more of the other reagents involved (such as the copper-glycine reagent) might diffuse poorly and limit the staining reaction.

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† Yamasaki and Narahashi[92] showed that the giant axons in the desheathed cockroach nerve are as sensitive to potassium and sodium as are mammalian axons.

An acetylcholine barrier was indicated by the work of Colhoun[12] who found that the isolated eserinized American cockroach nerve cord accumulated acetylcholine, yet none escaped into the perfusate unless the ganglia were mutilated, when about 10% of the total acetylcholine leaked out.

Another study[64] of penetration into the cockroach nerve cord was made with three organophosphates: TEPP, which is un-ionized; Tetram, a base with a pK_a of 8.4; and quaternarized Tetram, which is always ionized.

$$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$$

Free base (Amiton) whose oxalate salt is Tetram

$$(C_2H_5O)_2P(O)SCH_2CH_2\overset{+}{N}(C_2H_5)_3$$

Quaternarized Tetram

The index of penetration of these compounds was the resultant cholinesterase inhibition. When excised cords were compared with homogenates, the enzyme was a little less available in the excised cords, its unavailability being similar for all three compounds. But when excised cords were compared with intact cords (i.e., left *in situ*) three different effects were observed. With TEPP there was little difference in the availability of the enzyme of the two preparations at any pH. With quaternarized Tetram there was a profound difference independent of pH: the intact cord was little affected, the excised one was strongly inhibited. With Tetram, there was a pH-dependent effect: at high pH, when the compound would be mainly in the un-ionized free base form, there was a small difference between the excised and intact cords. At low pH, when the compound would be mainly in the ionized salt form, there was a much larger difference. These data therefore strongly suggest that there is an ion barrier around the intact nerve cord which is destroyed on removal of the cord. It is uncertain whether the destruction is on the ganglion surface, or whether the compounds penetrate through the cut ends, as seems more probable. This barrier distinguishes readily between the ionized and un-ionized forms of a single compound.

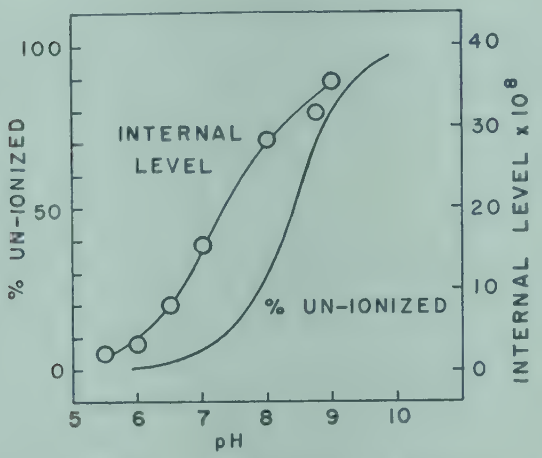

Fig. 5.1. Penetration of Tetram into the intact American cockroach nerve cord. The curve on the left indicates the molar concentration of Tetram inside the cord (scale on right of figure). The other curve is a dissociation curve plotted for a pK_a of 8.4 (scale on left of figure). The ordinates are on scales arbitrarily selected to make the curves the same height.

From the data on the variation of inhibition of the intact cord with pH, it was possible to calculate the amount of Tetram that penetrated, as shown in Fig. 5.1. Also shown is the dissociation curve of the compound which gives the per cent of the compound which is un-ionized at any given pH. Whey do the curves not overlap? There are two possible reasons, one likely and the other very unlikely. The first explanation is based on the assumption of an ion barrier, and considers that the shift of the "internal level" curve to the left is purely a function of the assay system. If we could measure the instantaneous rate at which external un-ionized Tetram penetrates the ion barrier immediately after the Tetram solution and the nerve cord are brought together, this rate should be strictly proportional to the external concentration of un-ionized Tetram. The plots of "per cent un-ionized" and "penetration rate" as a function of pH should overlap. If, on the contrary, we could only measure the internal level when equilibrium had been established between internal and external levels, then regardless of pH these two levels would be equal (assuming that the compound had equal affinity for the media on each side of the barrier), for the un-ionized form would diffuse across and re-establish equilibrium on the inside, and this would continue until the same concentration of both forms existed inside and out.

These two extreme conditions (i.e., instantaneous rate assay and equilibrium assay) should give results as illustrated in Fig. 5.2. Now in fact the cholinesterase assay system lies in between the extremes, but somewhat closer to the first extreme: the inhibition "response" is due to all the Tetram that diffuses across during the one hour incubation period, but gives "extra weight" to that which arrives first, for the first arrival has 1 hour in contact with the enzyme, whereas that which arrives 59 minutes later has only 1 minute in contact. Consequently, the observed "internal level" of Fig. 5.2 lies in between the extremes of Fig. 5.3.

An unlikely alternative is mentioned here, primarily, to add to the picture of possible penetration phenomena. Suppose the nerve cord has no ion barrier, but behaves as a simple lipid phase; the external medium,

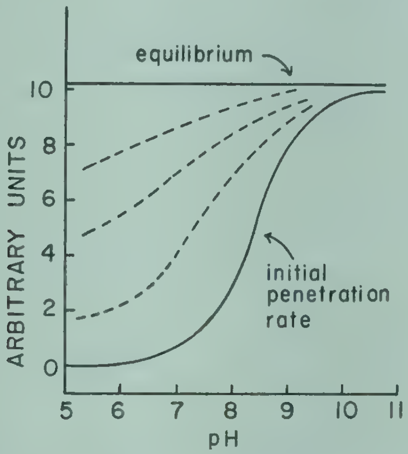

FIG. 5.2. Penetration of base, pK_a 8.4, through an ion-impermeable membrane. Similar media on both sides of the membrane are assumed. The intermediary curves are rough indications not based upon calculations. The solid sigmoid fits precisely over the dissociation curve for the base. Units are in terms of molar concentration.

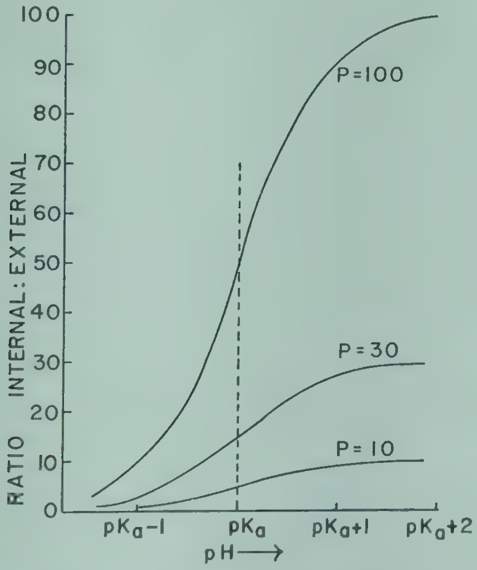

FIG. 5.3. Penetration of bases into a lipid from an aqueous phase. P = partition coefficient, internal (lipid) phase: external (aqueous) phase. Ratio on ordinate is that of molar concentrations.

the buffer, is the aqueous phase. Consider a base whose un-ionized form has a lipid: water partition coefficient of 10, and which is 95% ionized in the aqueous medium. Then at equilibrium there will be ten times more un-ionized form in the lipid than in the aqueous phase, and in the aqueous phase the un-ionized form will be one-twentieth of the total in that phase. Then the ratio of un-ionized form in lipid: un-ionized form in water: ionized form in water is about 10:1:20. Hence, the internal level will be 10/21 of the external. The internal level is thus a function of the partition coefficient of the un-ionized form and the difference between the pK_a of the base and the pH of the buffer, for this difference controls the degree of ionization in the aqueous phase. It can be shown* that at equilibrium the internal level, i.e., the level in the lipid phase, is the following fraction of the external level:

$$\frac{\text{internal level}}{\text{external level}} = \frac{P}{1 + \text{antilog}\,(pK_a - \text{pH})}$$

where P is the lipid-water partition coefficient. This expression is independent of the volumes of the two phases. From it, one can calculate the way in which internal concentration varies with pH for various values of P. Fig. 5.3 shows the curves for 3 values of P. The important point is that the inflexion points of all three sigmoids are at $\text{pH} = pK_a$. Now the experiment in Fig. 5.1 shows the inflexion point of the observed internal level shifted about 1 unit from the pK_a. It follows that simple equilibration phenomena cannot account for the Tetram findings. There is experimental confirmation of this view: the excised cords presented little barrier to Tetram penetration, only the intact cords were relatively impermeable. Clearly, Tetram can partition quite readily into nervous tissue once the barrier is damaged or (at the cut ends) removed.

The above discussion illustrates two ways in which penetration of ionizable compounds may be affected. An important point is that with either mechanism quaternary nitrogen compounds would be completely excluded, for they have no free base form with which to be in equilibrium.

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* As follows: the equilibrium conditions are $A \rightleftarrows B \rightleftarrows C$ where A and B are the concentrations of un-ionized form in lipid and water respectively, and C is the concentration of ionized form in water. Then

$$\frac{\text{internal level}}{\text{external level}} = \frac{A}{B + C} \cdots \text{(i)}.$$

Now $A/B = P$, and from the Henderson equation, $\text{pH} = pK_a - \log C/B$. The concentration units are now selected so that $B = 1$, and then $A = P$, and $C = \text{antilog}\,(pK_a - \text{pH})$. Substituting these values in (i), the equation given in the text above is obtained. (This formulation was developed in discussion with Dr. N. E. Good, to whom I would like to express my thanks.)

This consideration helps to explain why tertiary compounds may show effects strikingly different from their quaternary analogs, even though the pK_a of the tertiary compound is such as to assure 99.9% ionization at the prevailing pH. In special cases, however, a highly lipophilic substituent on the quaternary nitrogen may give a mildly lipophilic character to the whole molecule, as in pyridine-2-aldoxime dodecaiodide (page 157), and permit penetration into an aqueous phase.

The actual toxicant in schradan poisoning, hydroxymethyl schradan, behaves like an ionized compound as far as penetration of the cockroach nerve cord is concerned.[65] The compound is a potent anticholinesterase, but had little effect on the cholinesterase of the intact cord. If the cord was damaged or dissected out, the compound could then inhibit the cholinesterase extensively.

There is thus considerable evidence from different workers who have used various techniques that ions penetrate insect ganglia and nerves poorly and that, although variations exist in the phenomenon in different insect species and with respect to different ions, in general one must expect ionic toxicants to have much less effect than their nonionic analogs upon insect nervous systems. The implications for toxicity will be discussed in Chapter 9.

Two questions remain to be answered. (*1*) Is there a specifically located ion barrier? (*2*) How does it differ from (say) mammalian ion barriers?

The nerves and ganglia of all insects studied are invested with two sheaths.[18, 81, 87, 88] One is conspicuous, tough, and laminated, i.e., the neural lamella.* Inside it there is a second sheath or layer, by no means so clearly defined, consisting of nucleated cytoplasm containing mitochondria and tracheoles; this is the perilemma.† In the locust,[51] an external thin tracheolated membrane exists; in the American cockroach this may be absent[37, 38, 81] or it may be that it is fused with the neural lamella, as is claimed for the wasp by Edwards.[18]

Figure 5.4 shows an electron micrograph of a cockroach nerve in which the neural lamella and perilemma are clearly seen. In the cockroach ganglion, the neural lamella is much thicker and contains five clearly distinguishable layers: two thin homogenous layers sandwich three thick fibrous layers, distinguishable by the fact that their fibers run in different directions.[38]

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* It has been shown by Ashhurst[1] to consist of a collagen-type protein and neutral polysaccharide.

† This terminology is that of Hoyle[51] and Hess.[38] Wigglesworth[88] and Scharrer[73] refer to the perilemma as the "perineurium." Edwards *et al.*[18] refer to the whole neural lamella as the "lemnoblast basement membrane" and the underlying perilemma as the "lemnoblasts," while Hess[38] considers that only the innermost fine layer of the neural lamella is the basement membrane for the underlying cells.

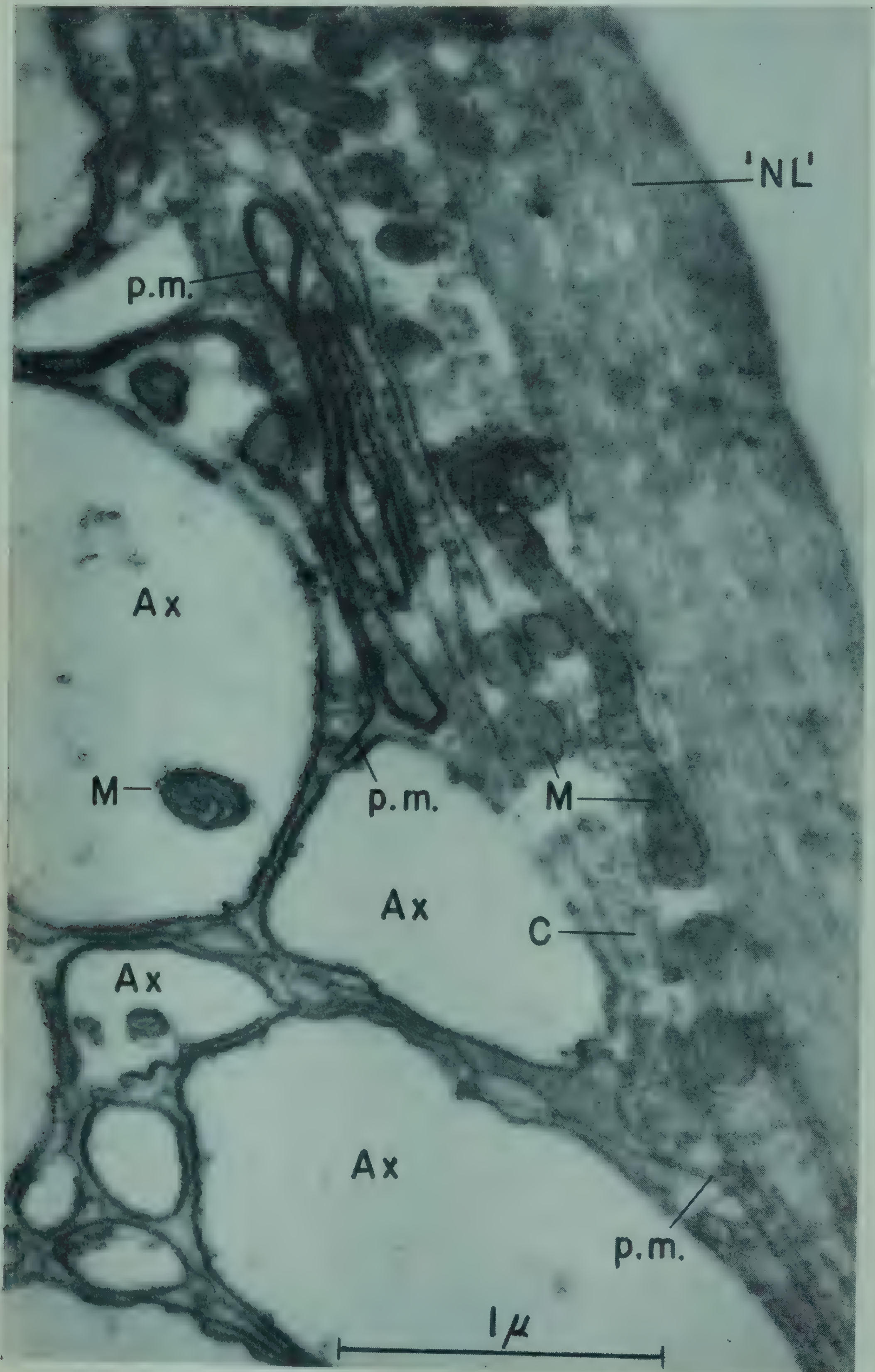
'NL'
p.m.
Ax
M
p.m.
M
Ax
C
Ax
Ax
p.m.
1 μ

Fig. 5.4

It seems probable that it is the perilemma which is the ion-impermeable sheath, the best evidence being that in histochemical studies reagents such as acetylthiocholine and silver ions penetrate the neural lamella readily but fail to penetrate the perilemma.[81, 86] In the locust, the outer tracheolated membrane may, when supplied with fresh tracheal air, account for some of the ion-impermeability.[51]

How do insects compare with other animals with regard to ionic protection of nerves? With respect to the protection of the central nervous system (i.e., the brain and spinal cord of mammals, and nerve cord ganglia of arthropods) and the motor and sensory nerves themselves, excluding the neuromuscular junctions, all animals have a considerable degree of ion protection. The perilemma of insect nerve bundles is however in some insects, such as the locust, a more effective ion barrier than the connective tissue sheath around the nerves of crustaceans and higher animals. The way in which all insects studied differ markedly from all mammals lies not in the ion barriers described above, but in the insects' lack of peripheral autonomic ganglia (which are present and ion-permeable in mammals) and in the relative ion-insensitivity of their neuromuscular junctions, as discussed in the next section.

In spite of the fact that Hoyle's discovery of a potassium barrier in the locust led to the idea of a generalized ion barrier, it would be dangerous to equate the insects' potassium barrier with that which excludes acetylcholine, ionized organophosphates, and hydroxymethyl schradan. For instance, there are important differences in the potassium barrier of the nerve sheaths of different species: that of the locust resists block by 140 m*M* potassium for 4 hours, that of the American cockroach for about 30 minutes.[51, 81] The cockroach nerve therefore resembles amphibian nerve in being only partially protected from potassium. Yet the ganglia of both insects are totally unaffected by acetylcholine.[36, 81] Furthermore, although the insensitivity of the cockroach ganglion to potassium is not influenced by severing attached small nerves,[81] and impermeability to acetylcholine is apparent in excised as well as in intact cockroach nerve cords (E. H. Colhoun, private communication), there is a very great difference between the

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FIG. 5.4. Electronmicrograph of an ultra thin section from the coxal region of the fifth leg nerve of an American cockroach. The nerve is enclosed by a connective tissue sheath, the neural lamella (NL) which measures about 0.7 to 1.0 *M* in thickness. Beneath the neural lamella is the perilemma, a cytoplasmic layer of dovetailed nucleated neuroglial cells rich in mitochondria (*M*) and cytoplasmic particles (*C*). These imbricated cells which are covered by plasma membranes (p.m.) extend into the nerve and surround the axons (Ax). Magnification *ca.* ×42,000. [Photograph and description kindly provided by Dr. C. L. Hannay and Dr. E. H. Colhoun from unpublished data.]

sensitivity of the cholinesterase of excised or damaged and of intact cockroach nerve cords to hydroxymethyl schradan[65] or to Tetram.[64]*

The situation then is not unlike that described above for crustacean ganglia, where excellent protection against large ions exists in spite of poor protection against metallic cations.

c. *Neuromuscular Transmission*

The sensitivity of the neuromuscular junction of the locust has been studied by Harlow.[36] It was insensitive to organophosphates (TEPP and paraoxon) even at 10^{-3} *M*, and to prostigmine at 10^{-2} *M*. However, eserine at concentrations greater than 10^{-3} *M* caused some reversible block. Acetylcholine was usually without effect, but occasionally induced a strong and transient tetanus. In such responsive preparations, subsequent applications of acetylcholine produced progressively smaller tetani, until "resistance" was virtually complete. The effect was not potentiated by eserine. Harlow concluded that neuromuscular transmission in the locust is not cholinergic. This view is confirmed by histochemical observations that cholinesterase is absent from the neuromuscular junction of the bug *Rhodnius*[86] and by assays of cockroach muscle which show the absence of choline acetylase, acetylcholine, or cholinesterase.[13] Possibly there is no chemical mediator at the junction, a condition which has been proven for a certain synapse in a crustacean ganglion.[24]

If this conclusion holds true for insects in general, we have here a tremendously important difference between insects and mammals, of particular interest in view of the fact that in many cases the acute effects of organophosphates on mammals are due to neuromuscular blockade. Although it might seem that as far as organophosphate toxicology is concerned we have no further interest in the insect neuromuscular junction, it is desirable for the sake of completeness to comment on the ion-sensitivity of the junction in insects as compared to other animals.

Microscopically,[18] the neural lamella of the nerve fuses at the neuromuscular junction with the muscle sarcolemma, i.e., the membrane surrounding the muscle fiber. The junction, as seen in the wasp, takes place in a groove in the muscle in which the axon lies; the groove is capped by perineurial tissue. Functionally, the neuromuscular junction is less sensitive to cations than is insect muscle, but more than is insect nerve; it is considerably less sensitive than the mammalian junction.[51, 52, 91] It seems likely that it shares the slight protection which muscle obtains from the investing tracheolated membrane, and also has some additional protection from its cap of perineurial tissue (which, when surrounding a nerve, is a highly effective ion barrier).

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* This argument is however not conclusive, for it is possible that cholinesterase in the ganglia can be very substantially inhibited without affecting nerve transmission. Indeed, Wigglesworth[86] finds in the bug *Rhodnius* that cholinesterase in the ganglion is confined to the interneuronal cytoplasm.

Let us summarize the differences in the insect and mammalian nervous systems which are of importance in organophosphate action. In both insects and mammals, the central nervous system is susceptible to un-ionized organophosphates but unaffected by ionic organophosphates (and in some cases, hydroxymethylschradan). Mammals have cholinergic peripheral autonomic ganglia which are sensitive to organophosphates; it seems that these are lacking in insects. Mammals have neuromuscular junctions which are cholinergic and ion-sensitive, and therefore are sensitive to both ionized and un-ionized organophosphates. Insects have noncholinergic neuromuscular junctions which are therefore unaffected by organophosphates.

It seems likely that aphids differ in some way from the above description;* this difference may well be found for other insects as yet uninvestigated. At present we may say that insects other than aphids are unaffected by ionic organophosphates because the only ion-sensitive part of their nervous system is not cholinergic. Mammals are affected at their ion-sensitive cholinergic neuromuscular junction.

d. Effects on Whole Nerve Cord

In the discussion so far the "classic" picture of action upon insects has been assumed, i.e., that the effects of organophosphates on the insect nervous system are due to cholinesterase inhibition, which in turn leads to interference with the acetylcholine relations in the system. An experiment by Sternburg *et al.*[75] throws doubt on the validity of this assumption. They took an isolated American cockroach nerve cord and bathed it in saline; high spontaneous activity was observed. The saline was replaced by 10^{-3} M TEPP in saline, and the block was prompt and completed, as expected. This sample of TEPP-saline was set aside; let us call it T. The preparation was then washed several times with fresh TEPP-saline, whereupon *normal spontaneous activity was restored.* If T was again placed in contact with the cord, excitation followed by block was observed.

This fascinating experiment was interpreted to mean that the blocking action of TEPP was caused not simply by cholinesterase inhibition, but by the action of some material whose production is induced (or enhanced) by TEPP. This material must be of limited availability in the cord, since only the first TEPP-saline addition was effective. A similar substance was found on DDT treatment or repeated electrical stimulation. Colhoun[13] reported that TEPP or DDT treatment of cockroaches led to the appearance in the blood of corpus cardiacum hormone, corpus allatum hormone, and three unknown factors. He suggested that these materials were produced as a result of nonspecific stress.

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* This is in view of their sensitivity to poisoning by schradan and Tetram. See page 332.

It is too early to evaluate these results, nor can we say whether the phenomenon is peculiar to insects. The possibility exists that we may have to re-evaluate our opinions on the way in which organophosphates affect insect nervous systems.

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CHAPTER 6

Effects in Mammals

Results of Poisoning

SYMPTOMS

On treatment with an acute dose of an anticholinesterase, the usual symptoms seen in all mammals are: defecation, urination, lacrimation, muscular twitching and fibrillation, muscular weakness followed by prostration, and convulsions which are usually clonic (i.e., rapid repetitive movements) and less often tonic (i.e., limbs stretched and rigid). Usually death is preceded by a clonic convulsion.

Smaller doses lead to corresponding but less severe symptoms, and occasionally to local paralysis, e.g., of the neck and foreleg in rabbits poisoned by parathion.[57] The appearance of symptoms is rapid with phosphates which do not require activation, and delayed in normal doses of the phosphorothionates and phosphoramidates, which require activation to become potent compounds. This delay depends upon the compound, the dose, the animal, and the route of administration; at the LD_{50} it is often of the order of 15–30 minutes.

At autopsy, the picture is similar whatever the method of administration. The diaphragm is elevated, with the lungs usually collapsed and ischemic, although occasionally they are congested. There is a spasm of the small intestine and the abdominal viscera with peritoneal effusion, and the spanchnic veins are engorged with dark venous blood. The right heart is distended and the left ventricle is often empty. Apart from the "venous" color of the arterial blood, the brain is generally normal in appearance, but there are sometimes a few petechiae (minute hemorrhagic spots) in the brain substance.[53]

Chronic poisoning in most cases produces protracted and milder forms of the symptoms given above, although convulsions may be absent. Salivation, diarrhea, and muscular weakness are typical.[18] Animals can become adapted to chronic poisoning: a fixed level of organophosphate, enough to cause severe symptoms, is eventually tolerated, even though it maintains a low cholinesterase level.[18, 154]

The detailed symptomology of poisoning has been reported for dimefox,[147] Systox,[58] Dipterex,[56, 65] Guthion,[69] Chlorthion, malathion, tetrapropyl dithionopyrophosphate,[15] HETP,[52] DFP,[86a, 108, 129] sarin, tabun,[112] parathion,[68] and schradan.[66]

Cases of poisoning in man have been reported for mipafox,[32] DFP,[84, 86a, 89] parathion,[81, 82] and sarin.[83]

CAUSE OF DEATH

It is generally agreed that asphyxiation is the ultimate cause of death in mammals.[17, 18, 53, 61] Perhaps the best evidence is that artificial respiration allows animals (rabbits) to survive doses of paraoxon which are normally fatal.[17] There are four different mechanisms which are involved, and there is some uncertainty about which is most important: (*a*) bronchoconstriction; (*b*) lowered blood pressure; (*c*) neuromuscular block of the respiratory muscles; and (*d*) failure of the respiratory center of the brain.

Douglas and Matthews[61] suggest that in cats poisoned by TEPP, factor (*d*) is of most significance; but in the rabbit treated with DFP or TEPP the peripheral effects (*a*), (*b*), and (*c*) seem to be primary.[54] [119] In the case of compounds such as schradan and dimefox, which contain dialkylamino groups, death is not accompanied by a reduction of brain cholinesterase[18, 147] and, therefore, it is unlikely that factor (*d*) is of importance in such compounds.

The classic work on this aspect is that of De Candole, *et al.*[53] done in 1953, in which seven compounds were studied against nine mammalian species. Their conclusion was: "The central failure seems to be the predominant feature in most instances, but the detailed picture varies with the species studied, the drug used, and the dosage administered. Thus, in the rabbit, bronchoconstriction is slight and develops slowly, while neuromuscular block may be severe at the diaphragm but is less marked at the chest muscles. In the cat, bronchoconstriction may be early and severe, and again, although neuromuscular block may occur at the diaphragm, the chest muscles retain their activity until central failure occurs. In the monkey, central failure appears to be the sole cause of cessation of ventilation, the bronchoconstriction and neuromuscular block being insignificant at the time of failure."

Artificial respiration will save atropinized animals from a lethal dose of e.g., paraoxon. Barnes has shown that a few minutes of artificial respiration may be all that is needed; the animal then recommences spontaneous respiration.[17] In the unanesthetized rabbit, the poisoning and saving can be repeatedly carried out, e.g., 10 times on one animal, although the artificial respiration requirement increases steadily. In the anesthetized rabbit, Barnes found a remarkable phenomenon: after the first dose and the first saving (by artificial respiration), the animals did not show any immediate response to subsequent doses. However, with repeated dosing, a slow failure of respiration and circulation occurred, leading to death in from 3 to 5 hours. But in spite of this phenomenon, the rabbits were rendered additionally sensitive to injected acetylcholine by each paraoxon injection—evidence that the inhibitor was having its expected effect upon cholinesterase. The increased tolerance of the anesthetized animals does not seem to be associated with reduced levels of acetylcholine in the blood.[20]

The remarkable difference between the behavior of anesthetized and unanesthetized rabbits suggests an interaction between anesthetization and organophosphate poisoning. Another interaction was reported by Holtz, *et al.*[94] who showed that pretreatment with paraoxon modified the anesthetic effects, in mice, of Avertin (tribromoethanol) and the barbiturate, Evipal. The effects were complex: a prolongation or a shortening of sleeping time was obtained, which was dependent upon the dose of organophosphate and the interval between administration of it and the anesthetic.

Several other physiological effects have been noted in phosphate poisoning. DFP in man greatly increased intestinal motility for several hours,[86] modified the electroencephalograph pattern,[84] and produced trains of potentials at the neuromuscular junction.[89] A detailed study of the complex effects of sarin and TEPP upon the cardiovascular system of the dog has been made by Daly.[42, 43] There was a marked variation of cardiovascular effects in different species: usually a profound fall in blood pressure was observed (often after a transient rise); but in the rat it was shown, for all cases studied (DFP, TEPP, and paraoxon), to cause an increase in pressure.[59] TEPP caused marked contraction of the spleen in the dog.[164]

From what has been said already, it seems safe to say that the chain of events leading to death in the mammal is (*a*) inhibition of cholinesterase, (*b*) acetylcholine accumulation, (*c*) disruption of nerve function, either centrally or peripherally, (*d*) respiratory failure, and (*e*) death by asphyxia. In order to support this concept, let us marshal a few pieces of evidence more fragmentary than those given before:

(1) Douglas and Paton[62] considered that the neuromuscular blockade caused by TEPP in the cat was exclusively due to the acetylcholine excess which it created: "no direct action of TEPP was detected or need be postulated to account for its neuromuscular effect."

(2) In rabbits poisoned with 0.3 mg./kg. of DFP, all the survivors had a substantial fraction of their brain cholinesterase uninhibited (average 24%, range 10–50%), whereas those killed by this dose had extremely low brain cholinesterase (maximum 4%).[137]

(3) That a lowered blood cholinesterase level does enhance the response to such compounds as acetylcholine has been shown without the use of inhibitors by replacing a part of the blood by saline.[98] Also, the partial replacement of normal blood by cholinesterase-free blood from a DFP-treated

animal (thus, ensuring that only blood cholinesterases were lacking) gave a potentiated response to acetylcholine. The response in question was the increase in blood pressure following intravenous acetylcholine administration.

Whether or not the sequence of events given above is correct, we may expect that the effectiveness of an organophosphate will be modified by the presence of enzymes which degrade it. In Chapter 9, it will be argued that a major cause of the low toxicity to mammals of such compounds as malathion, acethion, and dimethoate is the extremely rapid hydrolysis of these compounds in the animal. In Chapter 4, the existence of numerous enzymes capable of degrading toxic phosphates such as DFP, TEPP, and paraoxon has been shown, and we should expect that these enzymes will reduce the toxicity of the compounds.

CHOLINESTERASE AFTER POISONING

There is of course a tremendous wealth of data showing the response of cholinesterase in poisoned animals. If the enzymes *in vivo* behaved precisely as those *in vitro*, we might expect (on the basis of the factors discussed in Chapter 3) that after poisoning: (i) cholinesterase levels would fall fairly rapidly with phosphates and more slowly with phosphoramidates and phosphorothionates; (ii) a later recovery phase would occur within hours with dimethyl-substituted phosphates, within days with diethyl-substituted phosphates, and hardly at all with diisopropyl-substituted phosphates; (iii) whether or not recovery was complete would depend upon the extent of aging (page 106) that had occurred.

In general, these predictions are correct. But the following additional factors are important:

(*a*) Cholinesterase is steadily synthesized in the body, so that even after DFP poisoning a "recovery" is observed. Red cells recover at about 1% per day in man,[32, 86a] and plasma probably to the extent of about 25% in 10 days.[32] In lower animals, red cell replacement is faster, e.g., 10% per day in the rat.[88] In the DFP-poisoned rat, the pseudocholinesterase recovers with a half-life of 108 hours (brain) or 72 hours (other tissues), although in all cases the *in vitro* recovery half-life is over 250 hours.[50]

(*b*) Short term recoveries are sometimes unexpectedly rapid.[61, 141] This may be related to the reported presence in serum of a heat-stable "reactivating factor", i.e., an endogenous compound with PAM-like properties[141] (cf. page 101). Augustinsson's report[13] that tabun-inhibited cholinesterase can be reactivated by a plasma phosphatase may also be of significance in the recovery of blood enzymes. However, relatively rapid recovery is also found in tissues other than serum, e.g., brain, intestine, and salivary gland[91].

(*c*) For some entirely unknown reason, cholinesterases in poisoned ani-

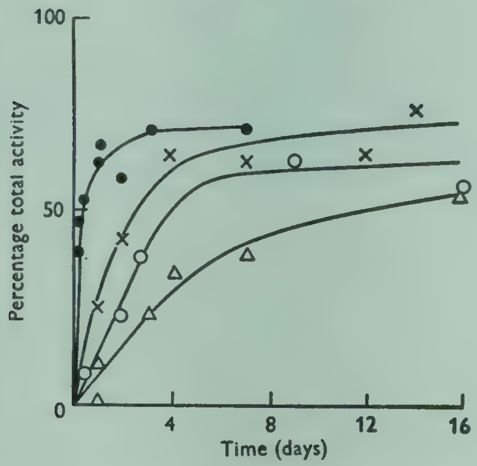

FIG. 6.1. The recovery of inhibited rat brain cholinesterase *in vivo*. Each point represents the mean brain time cholinesterase activity of two rats. Dimethyl (●), diethyl (×), di-*n*-propyl (○), diisopropyl and diisopropylamino (△) substituted phosphates were used in these experiments. From Davison.[50]

mals may recover to levels considerably above normal. This phenomenon was first observed by Koelle and Gilman,[109] was thoroughly examined by Locker and Siedeck,[117] and has been confirmed by Austin and Davies.[14]

(*d*) Besides the intrinsic selectivity to various esterases described in Chapter 3, another, physical, selectivity occurs: phosphoramidates do not inhibit cholinesterases in the central nervous system, probably because the inhibitor cannot penetrate the blood-brain barrier.[17, 67, 147]* The same is true for phosphates containing the ionic sulfonium,[172] or trimethylanilinium[30, 93] groups.

With these four factors in mind, the following figures of observed cholinesterase levels may be examined. Figure 6.1 shows the recovery of rat brain true cholinesterase *in vivo* after various treatments. Although the order of recovery rates fell in the expected order (methyl > ethyl > isopropyl) the isopropyl recovery was unexpectedly rapid. Also with every compound the recovery curve was biphasic: an initial rapid resurgence fell off when about 55% of the enzyme had recovered. Possibly this was due to aging having occurred with the other 45%; yet one would expect its extent to vary more than this amongst the various compounds. A similar biphasic curve was reported for the recovery of erythrocyte and brain cholinesterase of the human, the rat, cat, dog, and rabbit following poisoning by paraoxon.[50]

At the other extreme, Fig. 6.2 shows the marked over-recovery of chicken plasma pseudocholinesterase, which was far greater with DFP than with sarin. With the same animal and poisons, quite another picture was seen in spinal cord pseudocholinesterase (Fig. 6.3); a single dose of sarin led, eventually, to recovery to normal levels, but a single dose of DFP permitted

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* After treatment of rats with radioactive schradan, radioactivity was discovered in the brain;[8a] this was probably caused by unaltered schradan rather than by actual inhibitor.

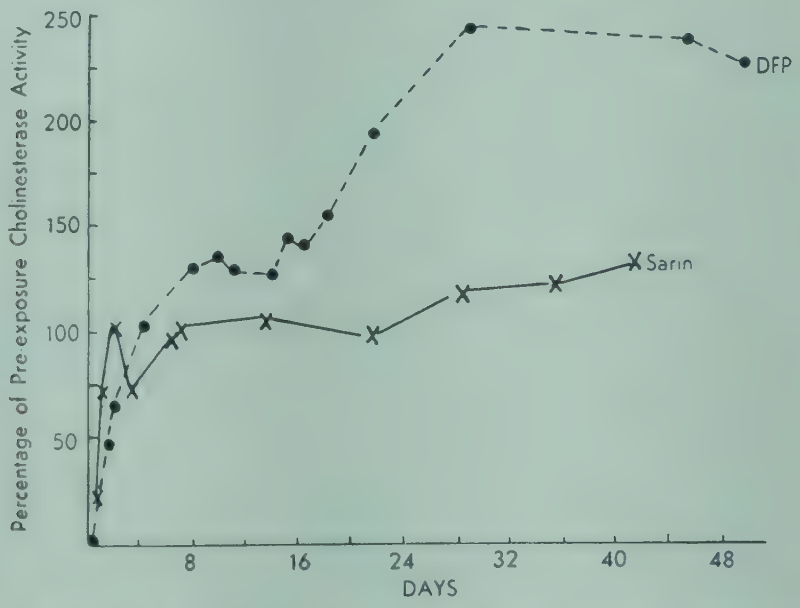


FIG. 6.2. Recovery of chicken plasma cholinesterase *in vivo* after treatment with DFP and sarin. From Austin and Davies.[14]

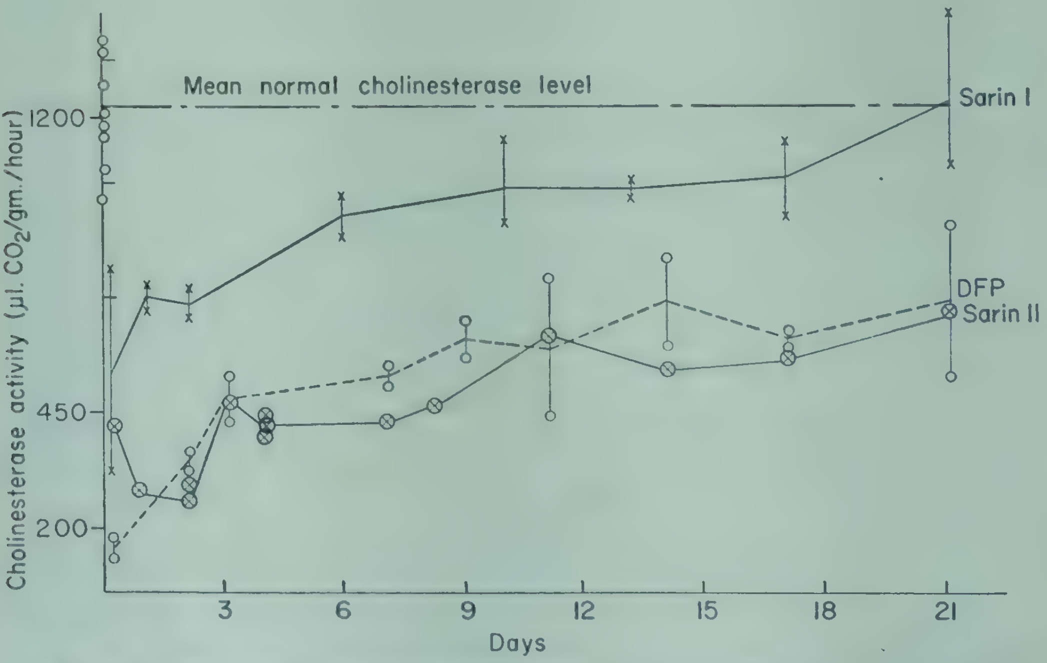


FIG. 6.3. Recovery of chicken spinal cord pseudocholinesterase *in vivo* after treatment with DFP and sarin. From Austin and Davies.[14]

only about 55% recovery. With true cholinesterase of chicken brain and spinal cord, and with pseudocholinesterase of brain, a recovery to 90–110% of normal was obtained with DFP or sarin in 21 days.

Locker and Siedeck[117] observed the phenomenon of the over-recovery of cholinesterases in the rat. With schradan, the effect was most marked in the brain (dosage 10 mg./kg., peak at 140% in 1 day); with dimefox, the effect was most marked in the brain and liver (dosage 1 mg./kg., peak at 130% in 3 days and still rising); with mipafox the effect was most marked in red cells and plasma (dosage 10 mg./kg., peak at about 125% in 7 days). However, with parathion, only plasma cholinesterase was affected, and the effect varied with dose, being most marked at low doses. Thus, at a dosage of 10 mg./kg., a peak at 145% was reached in 3 days; at a dosage of 0.1 mg./kg., the enzyme level was 165% at 6 days and steadily increasing. The authors therefore suggest that low levels of inhibitors stimulate enzyme synthesis.

Just as rat-brain pseudocholinesterase is unusually slow at reversal *in vitro* (page 100), so it is *in vivo*,[48] e.g., from 90% inhibition, the brain enzyme recovered to 65% in one day after paraoxon; all other pseudocholinesterases recovered to about 20%. Although this phenomenon was observed *in vivo* and *in vitro* to precisely the same extent, we cannot exclude the possibility that the brain lipid kept much paraoxon in contact with the enzyme and so induced rather extensive aging; both *in vivo* and *in vitro* the brain enzyme appeared to level off after recovery to about 50%, whereas the others all recovered completely. Whatever the explanation, the results are most clear-cut and serve as a warning against extrapolating results from one cholinesterase to another, no matter how closely related.

Data is also available[34, 142] for the recovery of brain and erythrocyte cholinesterase from sarin following vapor exposure: erythrocyte enzyme is back to normal after 40 days, brain recovers to 83% of normal in 60 days.

The short-term recovery of other esterases, as well as cholinesterase, has been studied in the rat by Seume *et al.*[165] In general, dimethyl phosphorylated esterases recovered more rapidly than the diethy, but in all cases the peak of inhibition was achieved at about 5 hours. The short-term inhibition of cholinesterase in various rat tissues after poisoning by symmetrical diethyl bis(dimethylamido) pyrophosphate is radically different, as shown in Fig. 6.4. One would conclude that this compound requires no activation, and yields a relatively irreversibly inhibited cholinesterase in all tissues. Casida *et al.*[32a] found that it was a weak antiesterase *in vitro*, and was not activated by liver slices. The data for brain in Fig. 6.4 demonstrate the poor penetration of the compound into the central nervous system, due to the dimethylamido group.

Data on cholinesterase levels in treated humans are available for schradan,[73, 155] parathion, dimefox,[73] malathion,[79] mipafox,[31] and sarin.[83]

In vivo results differ in an important way from the usual *in vitro* experiments, in which inhibitor is usually removed as soon as one wishes to examine recovery. *In vivo*, one has superimposed another factor upon the effects to be expected from such conditions: the persistence of the organophosphate, a factor characteristic of the whole molecule and not merely of the alkoxy groups. This may often account for compounds behaving differently *in vivo* yet similarly *in vitro*. The way in which cholinesterase inhibition parallels excretion rate for a single monkey poisoned with parathion is shown in Fig. 6.5. Assuming that a high excretion of paranitrophenol means a high blood level of parathion, it can be seen how inhibition at any time reflects the inhibitory level at that time. A similar state of affairs has been shown with Co-ral in the cow and goat.[113]

The fraction of *in vitro* inhibited cholinesterase which is reactivatable depends directly on the period of contact between inhibitor and enzyme.[46, 92]

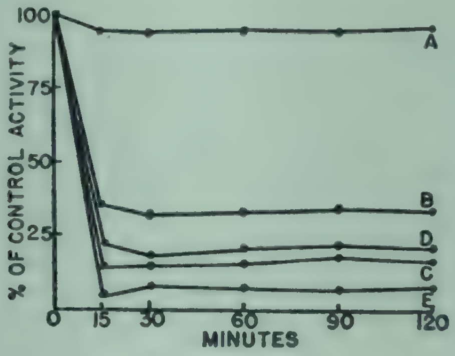


FIG. 6.4. Effect of symmetrical diethyl bis(dimethylamido) pyrophosphate upon rat cholinesterases *in vivo*. Curve *A*, brain; Curve *B*, skeletal muscle; Curve *C*, ileum; Curve *D*, submaxillary gland; and Curve *E*, serum. From DuBois *et al.*[67]

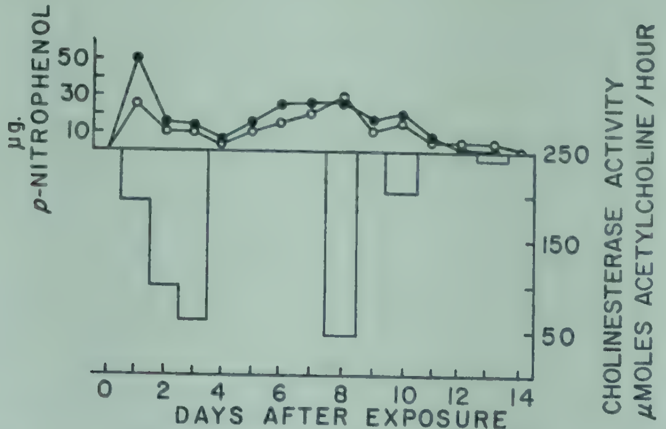


FIG. 6.5. Serum cholinesterase and urinary *p*-nitrophenol in a monkey poisoned dermally with parathion. From Waldman *et al.*[173]

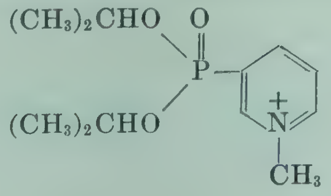
● = μg. per 24 hours.

○ = μg. per 100 ml. of urine.

The same is true *in vivo*. Vandekar and Heath[172] used a group of inhibitors all containing dimethoxy substituents; they found that those compounds which persisted in the body (or were activated to produce a persistent inhibitor) produced almost entirely the irreversibly inhibited enzyme, whereas compounds which did not persist gave inhibited enzyme which recovered spontaneously. By applying a normally nonpersistent inhibitor, methyl paraoxon, in such a way as to maintain a steady level in the blood (using prolonged infusion), these authors obtained the same pattern as with normally persistent inhibitors. Therefore the important variable is indeed the persistence itself. This important and elegant study points to one of the principal causes of discrepancy between *in vivo* and *in vitro* recovery rates. In fact, the authors showed that this series of compounds, which produced different patterns of recovery *in vivo*, all produced the same pattern *in vitro*, as tested by a technique in which all excess inhibitor was destroyed after a short incubation period. They also point out another conclusion: with two compounds of different persistence, "LD_{50} doses, although equilethal by definition, cannot be regarded as equitoxic": for survivors from the LD_{50} will be more susceptible to future poisoning in the case of the persistent inhibitor, which will yield irreversibly inhibited enzyme

A related phenomenon was studied by Davison.[50] Following one dose of the nonpersistent inhibitor dimethyl *p*-nitrophenyl phosphate in the rat, brain cholinesterase was inhibited about 90%; within one day it had recovered to about 30% inhibition, and little further recovery occurred thereafter. If similarly treated rats were given a second dose one day after the first, the cholinesterase repeated precisely the same pattern; and a third dose on the third day repeated the phenomenon again. Davison interpreted his data in terms of two fractions of cholinesterase, one easily inhibited and easily reversed (about 70%) and the rest which cannot be reversed. Supporting evidence for the hypothesis was that about 30% of whole brain cholinesterase is resistant to peptic digestion. When this pepsin-resistant fraction was treated with paraoxon, it recovered its activity at only 6% per day—very slowly compared with normal whole brain enzyme. This concept of Davison's does not appear to have been followed up, either by him or others.

As mentioned above, a number of anticholinesterases fail to produce substantial inhibition of brain cholinesterase *in vivo*, in spite of being excellent *in vitro* inhibitors. Such compounds often show selective action in ganglia. In 1952 Burgen and Chipman[31] prepared phosphopyristigmine:



a compound of low liposolubility due to its ionic pyridinium group. This compound did not inhibit brain cholinesterase *in vivo*. But they concluded that it was doubtful whether this failure was attributable to the failure to penetrate the blood-brain barrier, because the compound produced only localized inhibition when injected directly into the brain, thus by-passing the barrier. Inhibition was, however, obtained in the perfused superior cervical ganglion. On this rather limited data, these authors suggested that cholinesterase in the synaptic areas may be either (*a*) "outwardly directed" and, therefore, sensitive to inhibitors, e.g., in the sympathetic ganglia or (*b*) "inwardly directed" and insensitive to inhibitors, e.g., in the brain. By outwardly directed they implied that the enzyme was actually outside the neuronal cell.

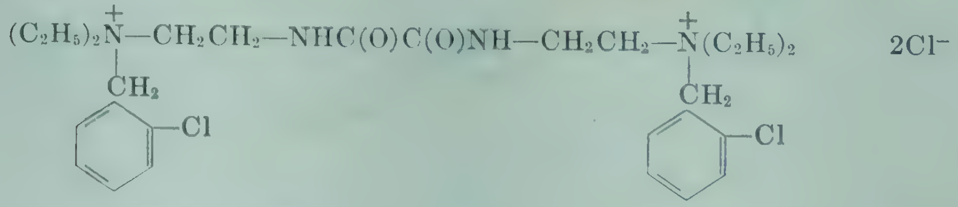
Koelle and Steiner[110] at first objected to Burgen and Chipman's argument, because they felt that if synaptic cholinesterase was internal, it would not be available for its presumed role of hydrolyzing acetylcholine. They therefore compared a base "217AO" and its quaternary analog 217MI.

$(C_2H_5O)_2P(O)SCH_2CH_2N(CH_3)_2$
217AO

$(C_2H_5O)_2P(O)SCH_2CH_2\overset{+}{N}(CH_3)_3$
217MI

Although 217AO was only a quarter as toxic as 217MI, it produced marked inhibition of the brain cholinesterase in intact rabbits, whereas the quaternary compound, 217MI, gave no such inhibition. If, however, the quaternary compound was injected into the ventricle of the brain, a clear inhibition of brain cholinesterase was found (even when the data was corrected for artifacts due to residual inhibitor in the tissue). To this extent, their data disagree with Burgen and Chipman's. Nevertheless, they did find that after brain injection of the quaternary compound, it was still present up to 4 days after treatment even though active cholinesterase was still present. This observation suggests that within nervous tissue at least a portion of the cholinesterase is unavailable to ionized compounds.

Later, Koelle[106] suggested that perhaps the internal enzyme was indeed a genuine fraction and represented a reserve of enzyme, whereas the external was truly a functional enzyme. He carried out experiments with the diquaternary compound, ambenonium chloride:



This compound reversibly inhibits cholinesterase and thus can protect the enzyme from irreversible inhibition by DFP. Using this property, Koelle employed a histochemical technique to show that ambenonium chloride failed to penetrate to the ciliary and stellate ganglia of the intact cat, but did penetrate to the motor end-plate. When a similar experiment was carried out with tissue slices (in which any protective membranes would be destroyed) the compound penetrated readily. The results support the Burgen-Chipman concept, that some cholinesterase exists inside the neurons and is unavailable to ionized agents.

This conception of two "geographical types" of cholinesterase has been extended by other authors. Wilson and Cohen,[179] working with intact crab nerve, applied the term "total" enzyme to the cholinesterase which could hydrolyze the tertiary analog of acetylcholine, (DMAE or dimethylaminoethyl acetate), and "external" enzyme to the cholinesterase which could hydrolyze acetylcholine. "Internal" enzyme was given by the difference between "total" and "external." This concept was based upon data which indicated that the intact crab nerve had at least one sheath around it which hindered the penetration of acetylcholine, but not that of the tertiary analog. In this terminology, "internal" enzyme is simply that which is inside the outermost protective barrier of the intact cord; there is not guarantee that all of their "internal" enzyme would be assayed by acetylcholine if one disrupted the outermost barrier.

Wilson and Cohen found that about 25% of the total enzyme was "internal," the rest was "external." However, quantitative estimates are extremely difficult because in assays on intact preparations one is never certain of the substrate concentration at the enzyme site, and one cannot simply saturate the system with substrate because of the phenomenon of excess substrate inhibition. Figure 6.6 shows some results of inhibition studies

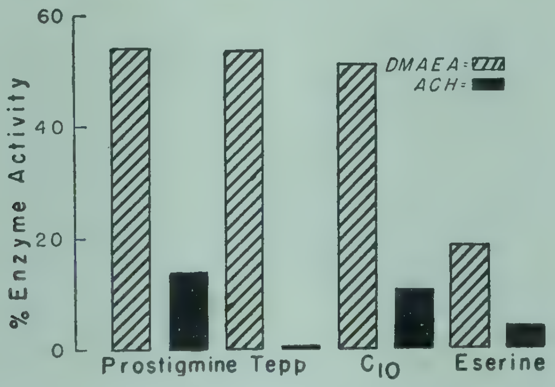


FIG. 6.6. Relative rates of hydrolysis by intact crab nerve of acetylcholine (ACH) and dimethylaminoethyl acetate (DMAEA), in the presence of various inhibitors. From Wilson and Cohen.[179]

Prostigmine: 4×10^{-3} M for 15 minutes.
TEPP: 3×10^{-2} M for 30 minutes.
C_{10} (decamethonium chloride): 3×10^{-2} M for 30 minutes.
Eserine: 3×10^{-2} M for 15 minutes.

(comparable data for DFP were not given). The authors stated that inhibitors fell into two classes: (*a*) prostigmine, TEPP, and decamethonium, which selectively inhibited "external" enzyme, and (*b*) DFP and eserine which inhibited "internal" and "external" enzyme. They felt that this explained the fact that the (*b*) compounds blocked nerve transmission, whereas the (*a*) compounds did not. However, the difference between prostigmine and decamethonium, on the one hand, and eserine, on the other, was not extremely striking (TEPP was indeed exceptional). The data are puzzling in that there seems to be no connection between ionization and penetration; thus eserine, which is 91% ionized at pH 7 (since the pK_a = 8.0)[143] penetrated readily, whereas TEPP failed to penetrate in spite of being un-ionized and capable of penetrating known ion barriers.[144] One would like to see such a study with more careful scrutiny of the time and concentration ranges involved; both of these factors appear to be randomly selected in Fig. 6.6. A more serious problem is the absence of data indicating that acetylcholine and its tertiary analog are hydrolyzed comparably by various esterases. Neither can one exclude the possibility that TEPP does penetrate the nerve, but is degraded there by a phosphatatse.

Yet another use of the term "internal" was made by Berkowitz,[23] also working with crab nerve. Here "internal cholinesterase" was the difference between the enzyme activity given by intact nerve ("external" enzyme) and that of the homogenized nerve. Acetylcholine was the substrate in both cases. Berkowitz found that about 40% of the total enzyme was "internal" in this sense, in fair agreement with Wilson and Cohen's figure of 25% for their "internal" fraction.

It is not easy to summarize these diverse studies, most of which use the term "internal" to mean different things. Clearly, ionized compounds penetrate poorly into the mammalian brain, inside the brain, and into crustacean axon. Probably there are two factors: (*a*) poor mobility of the compounds through the lipoidal ground-substance, and (*b*) the situation of a part of the cholinesterase inside the neuron, where it is protected by the neuronal membrane. In the case of crustaceans, perhaps a protective sheath may exist around the axon; but the two factors above may be enough to account for the fact that about 25–40% of the enzyme is unavailable to ionized compounds. Bases of pK_a's of 8 and over seem to behave surprisingly like un-ionized compounds, in spite of their being more than 90% ionized at pH 7 (e.g., eserine, dimethylaminoethyl acetate, Tetram). As a result, quaternerization of such bases impairs penetrability strikingly (cf. page 167). A contrary paradox exists in the case of phosphoramidates whose activated forms fail to penetrate the central nervous system in spite of having such low pK_a's that they will be totally un-ionized under physiological conditions. We have no information on whether the "blood-brain barrier" is responsible for this failure, or whether the compounds penetrate the ground-substance of the brain poorly.

There are basically two ways to find out the *in vivo* level of an enzyme at any given time: (i) perform an enzyme assay, usually on homogenates, sometimes on slices or whole tissues; (ii) use histochemical techniques.

The first method (when homogenates are used) has been objected to on the grounds of artifacts that may arise, particularly a redistribution during homogenization which may bring the inhibitor into contact with the enzyme from which it was separated *in vivo*.[137, 179] For instance, Kewitz[99] dried and extracted the tissue (mouse diaphragm) with chloroform before assay, and obtained during the first 10 hours after paraoxon treatment a higher cholinesterase level than in unextracted tissue (Fig. 6.7). Presumably, after this time the surplus paraoxon had been degraded by tissue enzymes. Blaber and Creasey,[26] however, have claimed that this procedure of Kewitz leads to spontaneous recovery of inhibited cholinesterase, and therefore gives artifactitious results.

Seume *et al.*[165] conducted rigorous tests and showed that in the case of various esterases in the rat tissues of four animals poisoned with malathion and parathion, there was no artifact produced by simple homogenization, as judged by comparisons of this simple procedure with a substrate-protection procedure. Hobbiger[93] showed that 3 hours after paraoxon poisoning there was no residual inhibitor in the brains of poisoned mice, as indicated by results of controls in which brains of poisoned and unpoisoned mice were homogenized together. It seems probable that substantial artifacts could arise only with compounds not requiring activation, and only

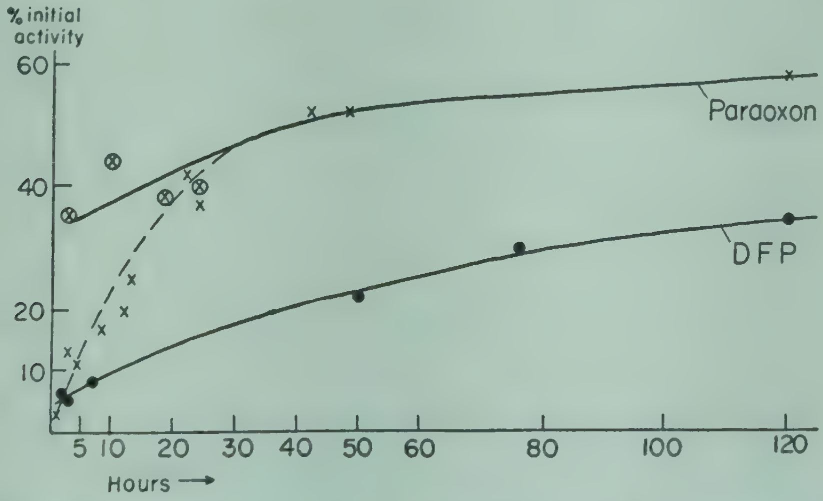


FIG. 6.7. Spontaneous recovery of acetylcholinesterase activity in the brain of mice after subcutaneous injection of paraoxon or DFP (LD_{50} dose). Chloroform extraction used (see text) for all except the paraoxon X——X data. From Kewitz and Nachmansohn.[100]

then within the first hours of treatment. The problem is discussed further in the next chapter, with regard to the insect (p. 244).

Method (i) has been used with preparations other than homogenates, e.g., with intact nerve preparations.[23, 179] This avoids possible homogenization artifacts, but has the disadvantage that the substrate level at the enzyme site is unknown, and is likely to vary in different parts of the preparation. This is particularly true when acetylcholine is the substrate. For instance, with intact crab nerve, the phenomenon of excess substrate inhibition cannot be shown: at 3×10^{-2} M substrate, the hydrolysis rate is the same as that at 3×10^{-3} M. Yet in homogenates, a sharp peak of activity is seen at about 10^{-3} M, and at 3×10^{-2} M the excess substrate causes about 50% inhibition.[179] Presumably, an outside concentration of 3×10^{-2} M does not achieve an inner concentration of more than 10^{-3} M: possibly it achieves far less. As for the tertiary analog of acetylcholine (dimethylaminoethyl acetate), although it appears to penetrate to areas unavailable to acetylcholine,[179] it is a base[143] with a pK of about 8.4 and therefore is 96% ionized at pH 7. We may therefore expect that its penetrability will be inferior to that of (say) TEPP.

In principle, the histochemical method offers advantages over other techniques for following esterase inhibition, particularly because of the lesser disturbance at the cellular level which is involved. A major drawback is the impossibility of any but the roughest quantitation of results. A comprehensive study by Gomori and Chessick[80] concluded that: "Differences amounting to less than ±50% cannot be recognized," and "Substrate preferences and inhibition effects overlap in such an irregular and unpredictable fashion as to defy attempts at classification." The unfortunate histochemist is severely limited in his available substrates, as they have to be potentially chromogenic as well. However, histochemical techniques are invaluable for studying very localized effects, such as enzyme levels at the motor endplate[22] and the penetrative capacities of ionized inhibitors.[106]

The question often arises, how useful are the various cholinesterase levels in indicating severity of poisoning? Let us first consider the case (not usual in accident problems) where one knows precisely the enzyme levels in the subject before poisoning. Usually enzyme levels on a particular subject may be studied only with erythrocyte and plasma cholinesterase. As Grob *et al.*[86a] point out, it is unlikely that the inhibition of these enzymes is causally related to symptoms. Rather, they reflect the state of affairs at more important loci, and these are probably the central nervous system, the neuromuscular junction, and certain ganglia. In all cases the vital enzyme is probably true cholinesterase, and it is therefore not surprising that plasma enzyme levels should be exceedingly poor indices of poisoning, particularly with fairly selective inhibitors such as DFP. Thus, plasma enzyme can be down to a few per cent of normal without severe symptoms.[86a] Red cell enzyme is more reliable, probably because of its similarity to essential enzyme. In the case of a human poisoned by DFP (3 mg./kg.), symptoms appeared when red cell cholinesterase fell below 70%, and were severe if it fell below 40%. However, at a lower dose rate, (1 mg./kg.), the level could be below 25% without symptoms.[86a] Similar irregularity was found in the rat, the dog, and the monkey treated with DFP.[107]

ACETYLCHOLINE AFTER POISONING

It is to be expected that a strong inhibition of cholinesterase should lead to increased levels of acetylcholine, since this substance is normally destroyed by the enzyme. Douglas and Paton[62] reported levels of acetylcholine of up to 0.04 μg./ml. in the blood of cats poisoned by TEPP (10 mg./kg.), and Stewart[170] found from 0.008 to 0.03 μg./ml. in the blood of rats poisoned by paraoxon (4 mg./kg.).

Barnes and Duff[20] showed with atropinized and artificially respirated rabbits, dogs, and cats, that blood acetylcholine was increased progressively with successive doses of paraoxon. With dogs, the highest level found was 0.03 μg./ml., even though total doses of up to 68 mg./kg. were given, spread over 6 injections, 30 minutes apart. In cats, up to 0.07 μg./ml. of acetylcholine were found under similar conditions. In rabbits, total doses of 2 mg./kg. (4 injections, 45 minutes apart) gave up to 0.043 μg./ml. of acetylcholine.

The source of the elevated blood acetylcholine levels is in dispute. Douglas and Paton[62] propose the bowel as the principal source in the cat. Barnes and Duff[20] used eviscerated cats to elucidate this point: acetylcholine production in paraoxon poisoning was indeed lower in such animals, but still attained levels up to one-half of those found in intact animals. Their data do not permit a more precise statement to be made. Certainly tissues other than the bowel make important contributions.

Acetylcholine is also elevated in the brain during organophosphate poisoning. Stewart[170] reported small but significant increases in the TEPP-treated rat, e.g., 5.98 μg./gm. as compared with control levels of 3.97, 3.60, and 3.67 μg./gm. in animals killed by asphyxia, strychnine, and chloroform, respectively. Similar increases were observed after treatment with DFP, eserine, and paraoxon. Michaelis *et al.*[127] carried out very carefully controlled experiments in the rabbit: one-half of the brain cortex was removed for a control assay, then the animal was injected with DFP (0.4 mg./kg.) and 30 minutes later the other half of the cortex was assayed. They found that the control levels of 0.66 μg./gm. were increased by DFP treatment to 1.33 μg./gm. in unatropinized animals; and controls of 0.50 increased to 1.09 in atropinized animals. Figure 6.8 shows results of similarly conducted experiments with longer times between dosing and assay. The highest level of acetylcholine was achieved about 4 hours after poisoning, and the level was still elevated at 18 hours. Normal levels were found between 24 and 26 hours.

DEMYELINATION

It has been observed in man that a few organophosphates, of which TOCP is the most familiar example, can, even with single doses, "lead to the development of a severe flaccid paralysis associated with demyelination both in the peripheral nerves and in the white matter of the spinal cord. Motor function is usually more disturbed in the feet and legs than in the arms, while sensation generally seems to be largely unaffected."[171] Recovery is often observed. About 14 days elapses between poisoning and onset of paralysis.

The classic case of TOCP poisoning occurred in 1930, when several thousand people in the U.S.A. were stricken with a flaccid paralysis, particularly of the lower limbs, about 10 days after drinking a contaminated ginger extract.[168] In one city with a population of 1500, there were 100 cases of paralysis. The ginger extract was used in every case for concocting an alcoholic "home brew," to beat prohibition. After some exemplary research, the contaminant was identified as TOCP, present to the extent of 2%.[169]

In the course of the research on this mysterious poisoning, some observations were made which have been confirmed often: humans, calves, chickens, and rabbits are paralyzed by TOCP, given by any route. The monkey and dog are almost unaffected by oral dosages, but are somewhat sensitive

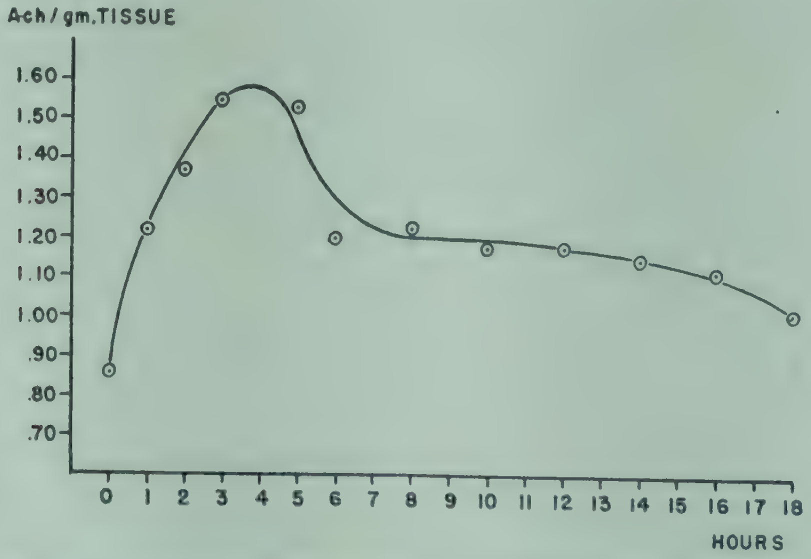


FIG. 6.8. Acetylcholine in rabbit brain cortex after DFP, 0.4 mg./kg. intravenously. From Michaelis *et al.*[127]

to subcutaneous or intramuscular dosage. However, in these animals, the paralytic dose is near the lethal dose, and it is difficult to obtain paralysis without subsequent death. Later work has shown that the rat is relatively insensitive to TOCP, and paralysis cannot be induced by any mode of administration.[49]

Bloch[27] proposed in 1941 that the paralysis was caused by acetylcholinesterase inhibition at the motor end-plate. However, Earl and Thompson[72] showed that TOCP was a very poor inhibitor of human acetylcholinesterase, yet a potent inhibitor of pseudocholinesterase. Since pseudocholinesterase is particularly associated in the nervous system with the white myelinated tracts,[30, 148] and can be shown histochemically in the Schwann cells of myelinated fibers,[105] Earl and Thompson[72] suggested that it was the inhibition of pseudocholinesterase that caused demyelination and, therefore, paralysis.

However, Davison[49] has shown that other selective inhibitors of this enzyme (schradan and its isopropyl analog) do not produce demyelination; and that demyelination cannot be produced in the rat, despite profound inhibition of its pseudocholinesterase. Austin and Davies[14] have also shown, using doses of DFP and sarin which produce identical enzyme inhibitions, that only DFP gave demyelination. The only remaining evidence in favor of Earl and Thompson's hypothesis is that all the demyelinating phosphates are selective inhibitors of pseudocholinesterase. Even this evidence has been further weakened by an extensive study on 25 aromatic compounds by Hine *et al.*[90] Only *o*-tolyl derivatives showed demyelination, and one of these, tri-*o*-tolyl borate, was not even a phosphate. There was absolutely no correlation between paralysis-producing activity and cholinesterase inhibition (plasma or true, fowl or human, *in vivo* or *in vitro*).

Recently Silver[167a] has shown that tri(*p*-ethylphenyl)phosphate produces a paralysis in hens which differs from that observed with TOCP: the legs are extended rather than flexed, symptoms occur more rapidly, and recovery is more frequent. The compound was stated not to be an anticholinesterase; however it was not clear whether it showed anticholinesterase activity *in vivo*. As pointed out above (p. 145), pure TOCP is not an inhibitor of cholinesterase *in vitro*, but is converted to one *in vivo*.

There is a possibility that TOCP demyelination is associated with an interference with vitamin E metabolism. Bloch and Hottinger[28] found that vitamin E antagonized paralysis of rabbits caused by TOCP. Draper *et al.*[63] showed that TOCP had particularly severe effects on vitamin E deficient lambs, and that these effects would be partially reduced by feeding alpha-tocopherol. They also noted that TOCP accelerated the deficiency symptoms of rats on a diet low in vitamin E, and that this effect was antagonized by feeding alpha-tocopherol. Myers and Simons[136] showed that chronic poisoning of rats with TOCP led to testicular degeneration, not unlike that caused by vitamin E deficiency. Other experiments showed, however, that TOCP effected testicular growth stimulated by testosterone acetate, yet not by testosterone. The authors consequently felt that the testicular effects of TOCP were attributable to its inhibition of aliesterase with consequent interference with testosterone metabolism. These results are far from conclusive. The fact that many of them are for the rat, an animal not subject to demyelination from TOCP poisoning, suggests that these vitamin E effects are unrelated to demyelination.

Of the compounds examined to date, the following have been shown to cause demyelination: TOCP, DFP, mipafox, diethyl phosphorofluoridate[19] and tri(ethylphenyl) phosphate.[167a] The following do not cause demyelination: sarin, tabun, soman, ethyl sarin,[14] Chlorthion, DDVP, Demeton, Diazinon, EPN, malathion, schradan,[71] tri-*m*-cresyl phosphate, and tri-*p*-cresyl phosphate.[5]

Mode of Administration

One of the reasons for the hazardous nature of organophosphates is that they can enter the body by so many routes. Accidental poisoning can occur not only by swallowing the compounds, but by contact with the skin or by breathing the vapor. This ease of entry has led to the use of many routes, experimentally. A great deal of the early work on DFP and its analogs employed respiratory intake, using units of "*Ct*," i.e., the product of concentration of the compound (in, say, milligrams per liter of air) and time of exposure of the test animal to the compound. This technique was used because the proposed use of the compounds was as "nerve gases" in chemical warfare. However, it is not particularly easy to prepare precise concentrations or to measure them, and the exposure chambers are also likely to be expensive.

Popular routes of administration nowadays are intravenous, intraperitoneal, oral, and dermal. Subcutaneous and intramuscular administrations are used less often. Some of the factors involved in the choice of route are as follows.

(*a*) *Intravenous:* a very rapid and, in the case of larger mammals, a very convenient technique. In animals smaller than the rabbit, it is not a particularly easy route because of difficulty in locating the vein reliably. In mice, prewarming the animal to dilate the veins is helpful, but even so mistakes are easy to make, and the solo worker needs some form of constraining apparatus. One is liable to expose the heart very suddenly to high levels of the poison, and thus the rate of injection is important.

(*b*) *Intraperitoneal:* the simplest procedure for small animals. Amateurs can become experts with an hour of practice. Uptake is rapid (e.g., 1 minute) but not so rapid as to expose the heart to sudden high levels. The compounds pass through the liver before reaching the heart, and this may be important if liver toxification or detoxification is critical. Thus, Adie[1] found that variations in liver sarinase level affected the toxicity of sarin when given intraperitoneally, but not when given subcutaneously.

(*c*) *Oral:* not a very difficult technique if the right instruments are used (for mice, ref.[130]; for rats, ref.[139]). However, uptake is slow, and very dependant upon vehicle and feeding status of the animals. It is best used for chronic studies, and to simulate possible hazard situations. In acute studies, it is liable to give very variable results.

(*d*) *Dermal:* not a difficult, but a tiresome technique for small animals. Animals must be partially shaved and prevented from licking the place of application (in rats, a wide adhesive bandage around the whole body is convenient). Results are affected if the vehicle is such as to modify the properties of the skin, e.g., the detergent Thiosolve 8139 increased the dermal LD_{50} markedly.[67] Solvents which are convenient for their rapid evaporation (e.g., acetone) may be unsuitable for this reason. It may be necessary sometimes to use this route to simulate possible hazard conditions.

Whichever route is used, there is always a possibility that the vehicle selected will affect the results. Unfortunately, data upon the influence of vehicle is scarce; Table 6.1 presents a recent study on the topic. The data of this table also show what we would expect from the factors discussed above, that the order of toxicity of a given compound varies with route as

TABLE 6.1. EFFECT OF VEHICLE AND ROUTE OF ADMINISTRATION UPON ACUTE TOXICITY

Compound	Vehicle	Intravenous	Oral	Intraperitoneal	Subcutaneous
Malathion	EL[b]	184	560	215	—
	Vegetable oil	—	1400	695	—
	Gum arabic	453	—	—	—
	Propylene glycol	—	233	132	—
Parathion	EL	5.4	6.4	8.2	11.6
	Vegetable oil	—	16.9	14.4	13.6
	Propylene glycol	1.05	6.7	4.6	7.8
	Mucin	—	11.6	8.8	—
Methyl parathion	EL	14.2	17.1	18.5	—
	Propylene glycol	2.3	18.5	8.6	18.0
	Mucin	—	58	32	—
Paraoxon	EL	0.39	—	1.65	—
Systox	H_2O	3.9	7.9	4.6	—
	EL	—	6.4	4.7	—

[a] Figures are acute LD_{50}'s. Results of Rosival *et al.*[160]

[b] *ERYFOR EL*, "a domestic emulgator."

follows: intravenous > intraperitoneal > oral ≧ subcutaneous. Dermal toxicities will usually be least (i.e., LD_{50} highest). Probably inhalation toxicities on a milligrams-per-kilogram basis would be comparable with intraperitoneal; but in fact the inhalation data cannot easily be expressed in this way.

De Candole and McPhail[55] studied the importance of the rate of injection, and found that the LD_{50} for sarin against mice was 0.1 mg./kg. for injections lasting 1 second, 0.14 for injections lasting 10 seconds, and 0.16 for injections lasting 20 second. Presumably, slow injection allows sarinase to work more effectively.

Degrading Enzymes

The role of degrading enzymes in determining toxicity has been studied by two workers. Saunders,[163] working with TEPP, DFP, and paraoxon in the rabbit, injected doses at 5 minute intervals. For each compound he found a linear relationship between ED_{50} (dose to kill 50%) and ET_{50} (average time to death). For DFP and paraoxon these lines had a positive slope (ET_{50} on the x axis) i.e., a certain dose given over a course of 50 minutes was less effective than the same dose given over 10 minutes. The slope of the lines probably measured the rate of degradation of the poison, and was 0.8 μg./kg./minute for DFP and 0.5 for TEPP. For paraoxon, a zero slope was recorded, suggesting (very surprisingly) that degradation was not important. The interpretation is however open to criticism because it presupposes that apart from degradation, it should make no difference in toxicity if a given amount of inhibitor is presented rapidly or slowly to the active site. This is probably not true.[172]

Adie[1] studied the importance of sarinase levels in determining the toxicity of sarin to rabbits. With intraperitoneal injections, there was a clear negative correlation between liver sarinase and susceptibility to poisoning: a twofold variation in sarinase was associated with an eightfold variation in susceptibility. However, there was no correlation between plasma sarinase level and susceptibility. When the subcutaneous route was used, neither liver nor plasma sarinase levels were correlated with susceptibility. These findings suggest that some other factor or factors of importance in determining susceptibility vary quite widely in the population, and only when intraperitoneal injection is used does the liver sarinase become overwhelmingly important.

Protection and Therapy

A great variety of agents are effective against organophosphate poisoning. Most agents are at their best in a prophylactic (protecting) role, i.e., given before the phosphate; but most are of at least some use therapeutically, i.e., given after the phosphate. The agents fall into five distinct functional classes, with apparently no overlap between classes: (*a*) Those which antagonize the effects of the excessive acetylcholine produced in poisoning. The best known is atropine. (*b*) Those which accelerate the reversal of the inhibited cholinesterase. The best known is 2-PAM. (*c*) Those which stimulate synthesis of degrading enzymes. (*d*) Those which protect cholinesterase from inhibition by combining reversibly with it. (*e*) Those which protect against phosphorothionates and phosphoramidates by blocking the enzymes responsible for activating these compounds. In general, the effectiveness of any protecting agent varies widely with different organophosphates and different species. In some cases however, a rough classification of effects is possible, based on the nature of the alkoxy substituents on the phosphate or its liposolubility.

ANTAGONISM TO ACETYLCHOLINE

As the "lesion" immediately preceding death in organophosphate poisoning is an excess of acetylcholine, a reasonable therapeutic procedure is to give a drug which antagonizes acetylcholine. A number of such drugs exist, and it is presumed that they act by competing with acetylcholine for some hypothetical receptor or receptors. Unfortunately, acetylcholine excess occurs at various places, central (e.g., in the brain), ganglionic, and terminal (e.g., the neuromuscular junction); in fact, wherever acetylcholine is a mediator (Chapter 1) one may get an excess of the compound in poisoning. But the drugs which antagonize acetylcholine are fairly specific in their action; thus atropine is active only in the central nervous system and ganglia, curare and pentamethonium are active only at the neuromuscular junction, and hexamethonium is active only in ganglia. The loci given here are those at which these drugs exert their principal action; small effects on other areas are often found.

The use of atropine was first suggested in 1942 by Barrett *et al.*,[21] who found that it prolonged life in rabbits poisoned by DFP, but failed to save the animals. Modell and Krop[128] found atropine alone fairly effective for cats poisoned by DFP. For example, 10 mg./kg. of atropine given 5 minutes before DFP allowed 86% of the animals to survive a 7 mg./kg. dose of DFP, i.e., 1.4 times the LD_{100}. The higher the DFP dose, the more atropine was needed. The atropine was given at various times from 5 minutes before to 30 minutes after the DFP but without much corresponding variation in effectiveness.

De Candole and McPhail[55] found a very wide variation between species in their response to therapy with atropine. As Table 6.2 shows, atropine alone raised the LD_{50} of sarin 1000-fold for the monkey, but had a negligible effect in the mouse. The table also shows that the effectiveness of

TABLE 6.2. PROTECTION BY ATROPINE IN VARIOUS SPECIES[a, b]

Species	Ratio — $\frac{\text{LD}_{50}\text{ with atropine}}{\text{LD}_{50}\text{ without atropine}}$	
	Sarin	Paraoxon
Monkey	1000	50
Dog	150	10
Cat	10	2.5
Rabbit	3	3
Mouse	1.1	2.1

[a] Data of De Candole and McPhail.[55]

[b] Atropine 10 mg./kg.

atropine varies with the phosphate used: in the monkey, for instance, atropine was twenty times more effective against sarin than against paraoxon. Yet in the rabbit, it was equally effective for both phosphates. Presumably this variability is a reflection of variations (*a*) in the relative importance of (say) central and peripheral effects in different species, as described on page 176, and (*b*) in the capacity of different phosphates to inhibit (say) central as opposed to peripheral cholinesterase as a result of their different penetrative capacities and of the different distribution of enzymes capable of degrading them.

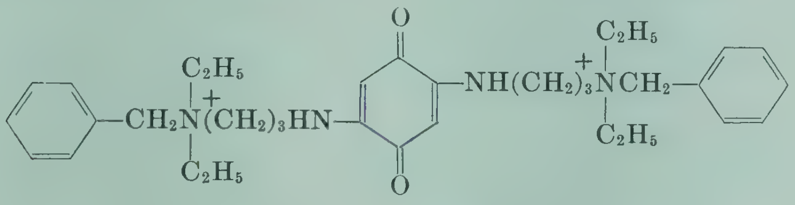
Although atropine is often the best available acetylcholine antagonist for protection against organophosphate poisoning, its effectiveness can be greatly extended if used along with another drug which can antagonize the neuromuscular disruption due to excess acetylcholine. A variety of agents have been proposed as adjuvants for atropine.

Magnesium was shown to be an effective adjuvant by McNamara *et al.*[125] in 1946. They used rabbits exposed to DFP vapor which killed 83% of the controls. Atropine (10 mg./kg. given before or after poisoning), reduced this figure to 53%; atropine and magnesium sulfate (400 mg./kg.) gave a figure of 47% if given 15 minutes before, or 13% if given immediately after the poisoning. Similar findings have been reported by others.[128] Magnesium by itself has negligible effect; its adjuvant action is due to its effectiveness against the neuromuscular blockade, which is unaffected by the atropine.[135]

Conley[35] studied the use of a number of analeptics as adjuvants: these are drugs which are medullary stimulants used for temporary resuscitation of the dying by stimulating respiration (centrally) and increasing the blood pressure. They were without useful effect.

Pentamethonium [$(CH_3)_3N^+(CH_2)_5N^+(CH_3)_3$] was used by De Candole and McPhail.[55] If proper "nursing care" was given to mice which escaped the acute effects of sarin poisoning, the LD_{50} (which was 0.14 for sarin and 0.16 for sarin plus atropine) could be raised to 0.93 mg./kg.

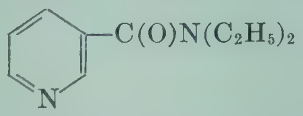
Mytolon (a curare-like drug) was recommended as an adjuvant by Lewis, *et al.*[115] for TEPP poisoning in mice and pigeons. Other combinations of a Mytolon-like drug and an atropine-like drug were effective, but not as effective as these two compounds. Using as criterion the amount (X) of drug needed to double the LD_{50} of TEPP, they found that in the mouse with atropine alone, X was greater than 160 mg./kg.; but with 0.013 mg./kg. of Mytolon added, X was 10 mg./kg. of atropine. Mytolon was found to be about three times better than *d*-tubocurarine.



Mytolon (benzoquinonium)

d-Tubocurarine and hexamethonium were compared as adjuvants to atropine therapy by Parkes and Sacra.[150] A variety of combinations of the three drugs was tried against each of the following: prostigmine, TEPP, Ro 30412, and Ro 30422. In consequence, their results are extremely complex, and show that the most effective therapy depends upon the anticholinesterase. In general, optimal effects were obtained with all three antagonists combined. Of the four anticholinesterases, only TEPP is un-ionized. Yet there was no obvious correlation between ionization of the anticholinesterase and response to the various combinations of antagonists, although one would have expected the central aspects of poisoning, which are atropine-antagonized, to be less important in the ionized compounds.

Coramine has been used as an adjuvant to atropine by DiStefano *et al.*[60]



Coramine (nikethamide)

(Coramine is a respiratory stimulant which acts upon the central nervous system to produce convulsions. It is used in the treatment of schizophrenia and narcotic poisoning.) For the male rat, the oral LD_{50} of EPN was raised 2.2 times by 90 mg./kg. of atropine given orally immediately after poisoning, and 3.6 times by atropine plus 120 mg./kg. of Coramine.

Reversal of Inhibited Cholinesterase

The primary effect of organophosphates is to block cholinesterase, therefore agents which can reverse this blockade might well be expected to alleviate the poisoning. The requirements for a reversing agent *in vitro* have been discussed in Chapter 3; for *in vivo* effectiveness the problems of metabolism and penetration to the site of action are additional factors.

The antidotal properties of 2-PAM or pyridine-2-aldoxime methiodide (page 102) were first reported by Kewitz and Wilson in 1956.[101] When used by itself, the effects are relatively small, and are dependent upon the organophosphate involved (Table 6.3).

The effects of 2-PAM are potentiated by atropine. Kewitz, *et al.*[102] found that the administration of 10 mg./kg. of atropine with 90 mg./kg. of 2-PAM gave complete protection against 10 times the LD_{50} of paraoxon in mice. With DFP treatment, protection against 5 times the LD_{50} was found. In these experiments, the atropine was given 30 minutes before the phosphate, and the 2-PAM, 1–2 minutes after the phosphate. Table 6.4 shows comparable results from Hobbiger,[93] in which the remarkable synergism—and the variation with phosphate used—is evident. Less dramatic synergism was reported by Wilson[178] for sarin: 2-PAM plus atropine only doubled the LD_{50} in mice. The results of Table 6.4 show that when this combination of antidotes is used, there is no simple connection between antidotal effectiveness and the dialkyl substituents of the phosphate. Thus, wide variation was found in the four compounds (1, 2, 4, and 5) which would give diethyl phosphorylated cholinesterase. Presumably, this is because of the different sites in the body attacked by these four compounds. But there was no correlation between antidotal effectiveness and ionization of the phosphate (4 and 5 of Table 6.4 are ionized); one might have expected ionization to be crucial in determining the site of action.

TABLE 6.3. PROTECTION OF MICE BY 2-PAM[a]

Organophosphate	2-PAM (mg./kg.)	Times increase in LD_{50}	References
Paraoxon	30	1.4	Kewitz *et al.*[102]
Paraoxon	75	1.6	King and Poulsen[103]
Paraoxon	25	2–4	Hobbiger[93]
Sarin	100	1.5	Loomis[118]
Sarin	90	1.2	Wilson and Sondheimer[180]
TEPP	90	1.8	Wilson and Sondheimer[180]
TEPP	25	2	Hobbiger[93]
DFP	30	1.3	Kewitz *et al.*[102]
DFP	75	1.1	King and Poulsen[103]
DFP	25	1–2	Hobbiger[93]
Schradan	50	1	Kewitz *et al.*[102]

[a] Data approximated from the references indicated.

Table 6.4. Protection of Mice by 2-PAM with Atropine

Organophosphate	Times increase in "lethal dose"[a]		
	2-PAM alone	Atropine alone	Atropine plus 2-PAM
1. TEPP	2	1–2	32
2. Paraoxon	2–4	2	128
3. DFP	1–2	2	16–32
4. Ro 30422	2	1–2	8–16
5. Ro 30340	8	1–2	32–64
6. Ro 30351	1–2	1–2	8

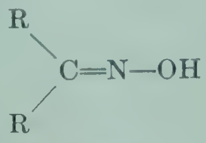
[a] Times are in minutes. Organophosphate injected subcutaneously at zero time. Atropine and 2-PAM given intraperitoneally. Atropine 50 mg./kg. at $t = -30$; 2-PAM 20 mg./kg. at $t = -5$. Data of Hobbiger.[93]

Wilson and Sondheimer[180] also reported the effects of combined 2-PAM and atropine treatment in mice. For TEPP, the LD_{50} was increased 9.3 times; for sarin and tabun only about 2 times; and for parathion, not at all. (But the extraordinarily high LD_{50} of 85 mg./kg. was given for parathion alone, cf. 5–10 mg./kg. of DuBois *et al.*[68]) Brown *et al.*[29] showed that combined 2-PAM and atropine treatment was effective in relieving sarin or tabun poisoning of the dog (2-PAM alone was ineffective). Bethe *et al.*[25] used these agents plus artificial respiration in the guinea pig to increase the LD_{50} of paraoxon 100 times, of parathion 70 times, and of DFP 21 times.

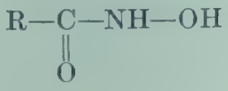
2-PAM has already come into medical use for treatment of accidental poisoning. A report[138] from Japan showed excellent effects of up to 1 gm. given intravenously after parathion poisoning: relief of pain and recovery of erythrocyte cholinesterase were both remarkably rapid, occurring within a few minutes. Serum cholinesterase was effected very little. No side effects of any significance were noted. These workers felt that atropine medication was not necessary now that 2-PAM is available.

In an attempt to overcome the relative ineffectiveness of 2-PAM in restoring brain cholinesterase, probably consequent upon its very low liposolubility, Wilson[177] prepared PAD, pyridine-2-aldoxime dodecaiodide; this is an analog of 2-PAM containing a "tail" of 12 carbon atoms attached to the quaternary nitrogen, in place of 2-PAM's single carbon "tail." PAD is about 50,000 times more soluble than 2-PAM in chloroform, and 200 times less soluble in water (unfortunately, partitioning data are not available). It is about one-third as active as 2-PAM as an *in vitro* reactivator of cholinesterase. By using combinations of 2-PAM, PAD, and atropine, the LD_{50} of sarin was increased about 3.4 times[177] as compared with 2 times for 2-PAM and atropine.[178]

Although 2-PAM has perhaps been the most popular oxime, a great deal of data has been published from various Chemical Defence groups concerning the toxicity and phosphate-antagonizing action of other oximes and hydroxamic acids.



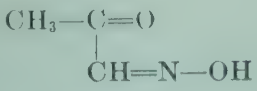
Oxime



$$\mathrm{R{-}C({=}O){-}NH{-}OH}$$

Hydroxamic acid

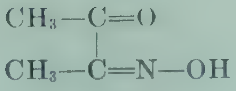
Askew[10] studied 23 oximes and 9 hydroxamic acids, and found 8 to be of use, i.e., giving about fivefold increases in LD_{50}'s. Two oximes, MINA and DAM, were studied in detail, and have also been studied by several other workers.



$$\mathrm{CH_3{-}C({=}O){-}CH{=}N{-}OH}$$

MINA

Monoisonitrosoacetone



$$\mathrm{CH_3{-}C({=}O){-}C(CH_3){=}N{-}OH}$$

DAM

Diacetylmonoxime

MINA is a good reactivator of sarin-inhibited cholinesterase, but is rather toxic; DAM is a poor reactivator but is of low toxicity (maximum symptomless doses in the rat: MINA 30; DAM $>$ 150 mg./kg.). The effect of DAM with various mammals is shown in Table 6.5. Obviously there is an enormous variation with species and with organophosphate. MINA was similarly tested, but although as effective as equal doses of DAM, its higher toxicity made it generally less useful. The results of Table 6.5 are for prophylactic conditions, i.e., prior to the injection of oxime. Therapeutic conditions were also studied; the oxime was useful if given up to 6 minutes after the phosphate, but not so effective as under prophylactic conditions.

Dultz *et al.*[70] have reported a comprehensive study of the toxicity and sarin-antagonizing effectiveness of 22 oximes. Results for the most effec-

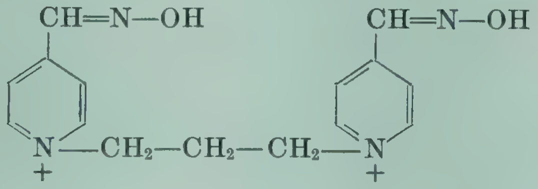
TABLE 6.5. PROTECTION BY DAM[a]

Species	Organophosphate	Times increase in LD_{50}
Mouse	Sarin	1.7
Guinea pig	Sarin	2
Rabbit	Sarin	1.6
Monkey	Sarin	approx. 3.0
Rat	Sarin	26.5
Rat	TEPP	2
Rat	DFP	1.3

[a] DAM given 15 minutes before phosphate. Dose: 150 mg./kg. against sarin, 200 mg./kg. for TEPP and DFP. Data from Askew.[10]

tive ones are summarized in Table 6.6. Apparently, these compounds are more effective for protecting rats than mice. The best is DAM. Askew[11] reported for the rat that atropine (17 mg./kg. given 15 minutes before poison) enhanced the protective action of MINA or DAM against sarin two to threefold. Atropine alone only increased the LD_{50} 1.3 times. In the monkey, atropine was somewhat more effective than in the rat against sarin (LD_{50} increased 3 times). Combined with MINA (20 mg./kg.) the LD_{50} was increased 14 times, with DAM (40 mg./kg.) it was increased 9 times.

Poziomek *et al.*[152] reported in 1958 on a new group of oximes far superior to 2-PAM: the molecule contained two PAM-like substituents linked by a methylenic chain. Berry *et al.*[24] made 30 such compounds. The best two were 1,3-di(4-hydroxyiminomethylpyridinium)propane dibromide and 1,6-di(hydroxyiminomethylpyridinium)hexane dibromide. Both raised the LD_{50} of TEPP to mice about a hundredfold, as compared to a fivefold increase for 2-PAM.



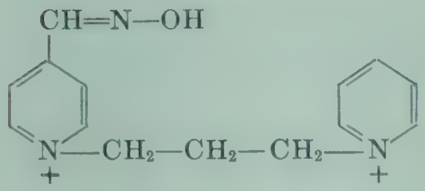
1,3-Di(4-hydroxyiminomethylpyridinium)propane

They were also reported to be better than 2-PAM against sarin poisoning (no data given). It seems that it is not essential to have oxime substituents on each end; for instance, the following compound raised the LD_{50} of TEPP 40 times:

TABLE 6.6. TOXICITY AND SARIN-ANTAGONIZING EFFECT OF OXIMES

Compound	Mice			Rats	
	LD_{50} of oxime mg./kg.	Prophylactic dose mg./kg.	Increase in LD_{50} of sarin[a]	Prophylactic dose mg./kg.	Increase in LD_{50} of sarin[a]
α-Oximino acetamide	4200	1000	>3	1000	7
Isonitrosoacetone (MINA)	150	75	1–2	30	5
Trioximinopropane	200	100	1	100	>2
α-Oximino malonamide	5000	500	2	500	<5
Diacetylmonoxime (DAM)	900	200	3	200	20
2-Oximino-3-pentanone	350	120	3	100	>5
Pyridine-2-aldoxime Methiodide (2-PAM)	190	75	1	75	1

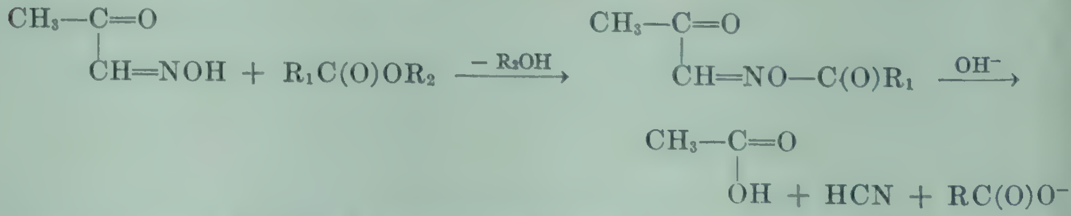
[a] Approximations from authors' data. Data of Dultz *et al.*[70]



1-(4-Hydroxyiminomethylpyridinium)-3-pyridinium propane

We will now consider the problems of the toxicity and metabolism of oximes. Studies on 2-PAM and DAM in man[95] showed no serious effects in doses up to 30 mg./kg. DAM caused few effects at up to 20 mg./kg., but at 30 mg./kg. produced transient coma and brain disturbances as indicated by the electroencephalogram. In mice, the LD_{50} of 2-PAM was 137 mg./kg., and the minimum lethal dose was 100 mg./kg.[102] Davies and Willey[47] examined the toxicity of the methanesulfonate salt of 2-PAM to various mammals (this salt was more soluble than the methiodide, which is usually used). A detailed account of the symptoms of poisoning was given. The toxicity by any given route was surprisingly similar for different mammals: for mouse, monkey, rat, rabbit, guinea pig, and dog the range for LD_{50}, intramuscularly, was 218 (dog) to 356 mg./kg. (monkey). From their comprehensive study, they concluded that 100 mg./kg. could be given to the rat, mouse, guinea pig, or monkey with a lethal probability of less than 1 in 1000. This was for the unatropinized animal.

Askew reports data for rats on the maximum nontoxic doses of 23 oximes.[10] Toxicities to mice are given in Table 6.6. Experiments in rats, rabbits, and dogs[12] suggested that the toxicity of MINA and DINA were due to the formation of hydrogen cyanide (but this is not so for DAM). For MINA, the reaction would be:



$$\begin{array}{l} CH_3\text{—}C{=}O \\ \quad | \\ \quad CH{=}NOH \end{array} + R_1C(O)OR_2 \xrightarrow{-\,R_2OH} \begin{array}{l} CH_3\text{—}C{=}O \\ \quad | \\ \quad CH{=}NO\text{—}C(O)R_1 \end{array} \xrightarrow{OH^-} \begin{array}{l} CH_3\text{—}C{=}O \\ \quad | \\ \quad OH \end{array} + HCN + RC(O)O^-$$

The compound $R_1C(O)R_2$ represents any kind of acylating agent, such as are known to be present in the body.

The persistence of DAM in intact animals was studied by Dultz *et al.*:[70] the half-life in the dog was 270 minutes and in the rat it was 137 minutes. Surprisingly enough, the persistence in the rat was not altered by nephrectomy. Hepatectomy, on the other hand, resulted in stable serum levels of DAM, and liver slices *in vitro* degraded DAM aerobically. Thus, liver degradation appears to be the major factor in DAM removal. Levels of DAM in 9 tissues were studied at various times: they varied little with tissue, except that blood contained 100 times more than the other tissues. Typical values: 30 minutes after 400 mg./kg., blood had 53 p.p.m.; liver, brain, heart, kidney, lung, spleen, muscle, and fat all had between 0.05 and 0.08 p.p.m.

Rutland[161] studied oxime levels in rat blood. His results for DAM disagree with those of Dultz *et al.*[70] in their nonlinearity and in their half-life value, which was only about 60 minutes (compare Dultz *et al.*, 137 minutes) Jager *et al.*[96] found, in man, that the half-life of DAM in serum was 7.2 hours, and for 2-PAM was 0.9 hours (both at 17 mg./kg.). 2-PAM appeared in the urine unchanged, but DAM did not. But within an hour, DAM appeared in the cerebrospinal fluid whereas 2-PAM did not. In the rat, nephrectomy severely reduced disappearance of 2-PAM from the blood, showing the importance of excretion in the rapid drop in the 2-PAM level of blood; but also liver minces degraded both compounds aerobically, suggesting the importance of liver metabolism.

Finally, we must consider the question: to what extent may we attribute the antidotal effectiveness of these compounds to their capacity to reactivate cholinesterase? Kewitz[99] showed that 2-PAM treatment of mice decreased the cholinesterase inhibition in the diaphragm, e.g., from 30% for paraoxon alone to 3% with 2-PAM treatment; from 93% with DFP alone to 72% with 2-PAM treatment; whereas with schradan (which is not antagonized by 2-PAM) no decrease of inhibition was found. Hobbiger[93] demonstrated comparable reactivations of erythrocyte cholinesterase from mice poisoned by compounds 1, 2, 4, and 5 of Table 6.4, i.e., those which yield a diethyl phosphorylated cholinesterase. But compounds 3 and 6, which yield a diisopropyl phosphorylated enzyme, gave "no consistent effect" (2-PAM *in vitro* was 30 times less effective against diisopropyl than against diethyl phosphorylated cholinesterase). However, no important reactivation of brain cholinesterase, *in vivo*, was found in poisoned mice, although 2-PAM reactivated brain cholinesterase, *in vitro*.[93, 100] This is to be expected from the ionic nature of 2-PAM. It is also compatible with the excellent synergism of atropine and 2-PAM; presumably, atropine attacks the central and 2-PAM the peripheral blockade.

These difficulties in correlating *in vitro* and *in vivo* effects of 2-PAM led Hobbiger[93] to doubt whether the antidotal effects could be entirely ascribed to cholinesterase reactivation. He cited other properties of 2-PAM: its neuromuscular blocking action and its own anticholinesterase action, and the possibility of its effect against actions of organophosphates other than cholinesterase inhibition. Brown *et al.*[29] also felt that the 2-PAM-induced improvement of respiration in atropinized dog poisoned by sarin or tabun was too abrupt to be attributed to enzyme reactivation.

Further evidence that 2-PAM and DAM may have a direct effect independent of cholinesterase reactivation comes from the work of Grob and

Johns upon humans.[85] The two oximes were effective in relieving neuromuscular block caused by schradan and by prostigmine and other carbamates. Yet *in vitro* cholinesterase inhibition by such compounds was not reversed by the agents, and indeed the authors showed that 2-PAM did not effect the cholinesterase of the patient poisoned with prostigmine, whereas it did improve the muscular strength and decrease fasciculations. A direct effect may also explain the finding by Bethe *et al.*[25] that 2-PAM alleviated poisoning of the guinea pig by the carbamate eserine.

It has been suggested above (page 103) that some of the effects of 2-PAM could be due to its catalysis of the nonenzymic hydrolysis of the phosphates. Kewitz *et al.*[102] report that 10^{-3} *M* 2-PAM at 25°C. and pH 7.8 hydrolyzes only 1% of 10^{-4} *M* paraoxon per hour. This implies that the effect is of negligible importance. On the other hand, in the case of tabun, Wilson and Sondheimer[180] suggest that the (very small) efficacy of 2-PAM in poisoning is attributable to its catalysis of hydrolysis. This was based on the observations that (*a*) 2-PAM *in vitro* did not reactivate tabun-inhibited cholinesterase and (*b*) 2-PAM *in vitro* at 10^{-3} *M* reduced the half-life of tabun (10^{-4} *M*) to 7–8 minutes, under aqueous conditions, pH 7.4, and 25°C. Although both these concentrations are probably higher than those encountered *in vivo*, the catalytic effect probably could account for the *in vivo* findings.

Rutland[161] attempted to evaluate the contribution of cholinesterase-reactivation in sarin-poisoned rats. *In vivo*, with the agents given before sarin, DAM was better than MINA in protecting brain cholinesterase, worse in protecting blood cholinesterase. But cholinesterase reactivation in brain (studied by administering the agent after cholinesterase was 90% inhibited) was fairly substantial with MINA (restored to 60% inhibition) and only slight with DAM and 2-PAM. In blood, all three agents restored the blood from its preagent inhibition of 88–97% up to about 70%. Rutland concluded that since DAM gave good protection of brain enzyme only when administered before the sarin, this protection must be attributed to its capacity to accelerate sarin destruction. In the case of MINA, on the other hand, at least a part of its protective effect was attributable to enzyme reactivation.

In general, agents will be useful therapeutically only if they have a strong reactivation effect, but may be of use prophylactically if they have only an inhibitor-degrading effect. Poor liposolubility and rapid metabolism are other factors which may account for the ineffectiveness of agents *in vivo*, which are excellent *in vitro*. We may expect that agents of low persistence will be particularly poor in phosphorothionate poisoning as a result of the lag period before the active anticholinesterase reaches high levels; and that agents of low liposolubility will be particularly poor against organophosphates which owe much of their toxicity to central effects.

Finally, the observation (page 105) that certain oximes can combine with some organophosphates *in vitro* to give potent anticholinesterases, has obvious implications for the search for good protective agents. The appropriate *in vivo* experiments to study this phenomenon have not yet been carried out.

Raising of Esterase Levels

It is to be expected that the toxicity of any organophosphate, X, will be reduced by treatments which raise either the level of "X-ase" (i.e., any X-degrading enzyme) or the level of X-inhibitable esterase. This last effect has two variants: the X-inhibitable esterase might be one whose inhibition was directly responsible for the toxic symptoms (e.g., brain true cholinesterase) or it might be a functionally unimportant enzyme which could, however, compete with some essential enzyme for phosphorylation by X, and in this way protect that essential enzyme.

In 1954, two papers from Kay's laboratory[16, 40] showed that aldrin treatment gave protection against parathion and TEPP, probably by an esterase-elevation effect. When aldrin was fed to or injected into rats, their serum esterase was remarkably increased, as shown in Fig. 6.9. Other data on single subcutaneous injections of aldrin showed that the esterase-increasing effect was linearly related to the logarithm of the dose. With single dosing, the esterase level was highest at 5 days and was then 30% above normal; the level returned to normal by 20 days. Five other chlorinated

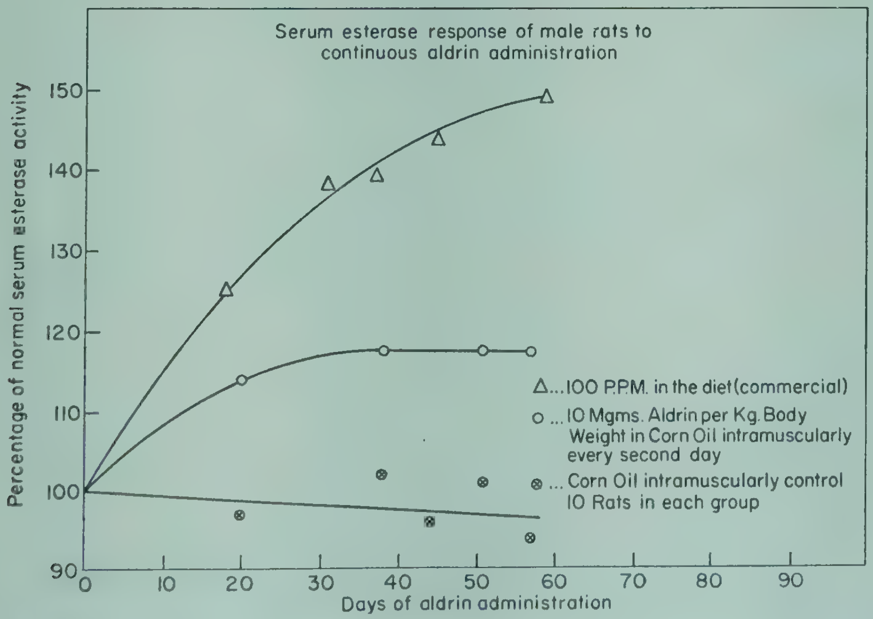


FIG. 6.9. Effect of aldrin feeding upon serum esterase levels in rats. From Crevier *et al.*[40]

hydrocarbons were studied and all of them increased the serum esterase level, as shown in Table 6.7.

Four days after acute oral treatment of female rats with chlorinated hydrocarbons, their susceptibilities to parathion poisoning were studied. Aldrin (30 mg./kg.) increased the LD_{50} 7.3 times, chlordane (200 mg./kg.) increased it 5.5 times, lindane (30 mg./kg.) increased it 2.5 times. Thus, although the data do not permit a precise statement, there is a rough correlation between the esterase-elevating effects (Table 6.7) and the parathion-protecting effects of these three compounds. When the parathion and aldrin were given simultaneously, very small protective effects were observed, as is to be expected from the progressive nature of the esterase-elevation effect. Aldrin (30 mg./kg.) was also found to protect against TEPP: four days after aldrin treatment, the LD_{50} of TEPP was increased fivefold. No protective action against eserine was found.

Before discussing the probability that esterase elevation is causally related to organophosphate protection, we must decide precisely which esterases were elevated. Phenyl benzoate was used for the routine "esterase" assay; but experiments were carried out to show that after aldrin treatment there was no increase in serum pseudocholinesterase (using benzoylcholine as substrate) or erythrocyte acetylcholinesterase (using acetyl-β-methylcholine as substrate), but there was an increase in triacetin hydrolyzing activity which closely paralleled the increase in phenyl benzoate hydrolyzing activity. On this basis, the authors felt that the "serum esterase" which was increased was aliesterase. Unfortunately we can no longer consider that there is a single entity which we can call "aliesterase," which is described as "an esterase of the type that acts preferentially on the simple aliphatic esters and glycerides."[153] Aldridge has disrupted this charmingly simple picture.[3, 6]

TABLE 6.7. ELEVATION OF RAT SERUM ESTERASE BY CHLORINATED HYDROCARBONS[a]

Compound[b]	Dose (mg./kg.)	% Increase in serum esterase[c]
Aldrin	30	30
Chlordane	225	29
Dieldrin	30	20
DDT	125	16
Heptachlor	45	10
Lindane (γ-BHC)	1800	10

[a] Data derived from Crevier *et al.*[40]

[b] Compounds given in single oral dose in corn oil.

[c] Levels are at 5 days after dose for Aldrin, 4 days for others.

Now Aldridge[3, 4] has shown that rat serum esterases are of two types, A and B, and that the A-type hydrolyzes paraoxon, which is of course the toxic agent in parathion poisoning; if Mounter's results for rabbit serum[131] are true of rat serum, A-esterase also hydrolyzes TEPP. We seem, then, to have a very attractive story emerging: the chlorinated hydrocarbons may stimulate the serum A-esterase level, which in turn protects against TEPP and paraoxon by hydrolyzing these compounds in the blood. The hypothesis readily explains why one cannot get eserine protection. It seems from the discussion of Ball *et al.*[16] that such is their hypothesis; but their actual conclusion suggests a confusion between the two possibilities mentioned at the beginning of this section, i.e., an increased phosphorylatable enzyme level which competes, or an increased hydrolyzing enzyme level which degrades. They say, "It seems more probable that the elevated aliesterase activity . . . provides a reservoir of esterase which competes for hydrolysis by organic phosphates, thus sparing some of the true cholinesterase."

Unfortunately it is impossible to guarantee that the rat serum enzyme which hydrolyzes phenyl benzoate and triacetin is an A-esterase. The terrible complexity of esterases in serum has been studied carefully by Aldridge,[3, 4, 6] but he used neither phenyl benzoate nor triacetin on rat serum. Tributyrin was used, but was barely hydrolyzed. In the rat pancreas and brain, triacetin hydrolysis was mainly the B-type (which would be inhibited by paraoxon, and would not hydrolyze it) although about 15% was the A-type. If such a distribution were found in serum, this partial activity in hydrolyzing paraoxon and TEPP could account for the results.

In summary, there is no direct proof that the esterase whose levels are elevated by aldrin is the A-type, although there is suggestive evidence and this would provide the neatest hypothesis. If it is not an A-esterase, it is almost certainly phosphorylatable and therefore could compete with a more vital enzyme (e.g., erythrocyte acetylcholinesterase) for phosphorylation by paraoxon. This alternative is very unlikely because it is difficult to conceive by this mechanism how a mere 30% increase in such an esterase could give a sevenfold protection against paraoxon.

Protection by prior treatment with a chlorinated hydrocarbon has also been shown by Neubert and Schaefer with mice.[140] They treated mice with α-hexachlorocyclohexane (α-BHC) at 35 mg./kg. and found, 4 days later, a protection against poisoning by paraoxon or schradan, but not against DFP or eserine. The α-BHC treatment had no effect upon cholinesterase levels. From their data, the LD_{50} seemed to be raised about 1.7-fold, a result which is in harmony with the results given above for the effect of the γ-isomer (lindane) upon parathion poisoning.

The authors concluded that the effect was due to a reactivation of inhibited cholinesterase by "physiological metabolites" which accumulate during BHC treatment. This conclusion was based primarily upon an elimination of the four other possible mechanisms, of which one was the degradative hypothesis similar to that advanced above. Neubert and Schaefer discounted this hypothesis on what seems a quite inadequate basis; they refer to their finding that when different paraoxon levels were given to mice, a proportional effect upon cholinesterase was not observed in all tissues. For example, plasma cholinesterase was not proportionately lowered. But surely it cannot be doubted that any effect that lowers the circulating level of paraoxon will reduce the lethality of a given dose of paraoxon.

As Neubert and Schaefer used α-BHC, and we have no data for the effect of this compound on A-esterase levels, we cannot reach a definite conclusion about the mechanism of its protective action. However, it seems very probable that all these chlorinated hydrocarbons exert their effects by the same mechanism, i.e., an increase in the A-esterase level.

BLOCKADE OF CHOLINESTERASE

As the organophosphates kill by blocking cholinesterase, it seems remarkable that one could protect against these compounds by prior block of the enzyme with another agent. But where the other agent produces a reversible blockade, such is the case.

Koster[111] showed that small doses of eserine could protect the cat against a usually fatal dose of DFP. As little as 0.01 mg./kg. of eserine increased the LD_{50} more than fourfold. Best effects were produced if the eserine was given a few minutes before the DFP; when several hours elapsed between injections the protection was much less. Quantities of eserine up to 0.25 mg./kg. were effective, although the LD_{50} of eserine by itself is only 0.66 mg./kg. The protective action of eserine along with atropine was quite striking: with 2 mg./kg. of eserine plus 0.3 mg./kg. of atropine (very low dose), protection against 30 times the LD_{50} of DFP was obtained.

The reverse experiment was tried: DFP was given before the eserine. In this case, potentiation rather than antagonism was found: 0.25 mg./kg. of eserine plus 1 mg./kg. of DFP killed 71% of the cats.

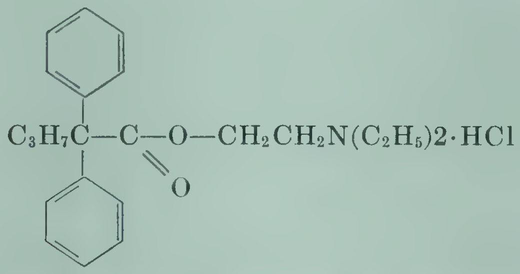
These observations have been extended to other phosphates in studies from DuBois' laboratory; with atropine and eserine the LD_{50}'s of symmetrical and unsymmetrical diethyl bis(dimethylamido)pyrophosphate were increased twelvefold and elevenfold, respectively; this was better than five times the effectiveness of the atropine alone.[67] The LD_{50} of schradan was raised elevenfold by eserine and atropine, as compared with fourfold by atropine alone.[66] The LD_{50} of parathion was raised eightfold by eserine and atropine, as compared with about twofold by either eserine or atropine alone; they noted, as did Koster, that if the eserine was given after—instead of before—the phosphate, additive rather than antagonistic effects were obtained.[162]

A weak protection by eserine against parathion poisoning was also reported by Wilhelmi and Domenjoz.[175]

Antagonism of Activating Enzymes

All the phosphorothionates and many of the phosphoramidates are poisonous only because the liver converts them into potent anticholinesterases. The activating enzymes responsible can be blocked by certain compounds, and as a result the toxicity of these activatable phosphorus compounds can be reduced.

The phenomenon was first observed in 1955, by Davison.[51] Having found that the *in vitro* activation of parathion and schradan was antagonized by SKF 525A, he carried out preliminary experiments which indicated that poisoning of rats by schradan, but not by parathion, was antagonized by SKF 525A (100 mg./kg., 20 minutes before the phosphate).



SKF 525A (β-diethylaminoethyl diphenylpropylacetate hydrochloride)

Later work with mice, by O'Brien and Davison,[146] showed that SKF 525A was the best of 12 agents studied for their capacity to antagonize schradan. The LD_{50} of schradan could be increased fourfold by simultaneous administration of SKF 525A (20 mg./kg. given intraperitoneally). There appeared to be two optimal periods for treatment: the antagonist could be given 1 hour before or simultaneously with the schradan. Even given 20 minutes after schradan, the antagonist conferred some protection. The puzzling failure of SKF 525A to antagonize parathion poisoning was confirmed. Several other phosphorus compounds were studied: the LD_{50} of Guthion was raised twofold, so the failure against parathion was not because of a general ineffectiveness against phosphorothionates.

Besides schrandan, four other compounds containing dialkylamino groups were studied. When the only potentially activatable group in the molecule was diethylamino, no antagonism to SKF 525A was found (this was true of Tetram, and the ethyl analog of schradan). The thiono isomer of Tetram was antagonized, presumably by an effect on the thionophosphate activating system.

A parallel was observed between the effects on schradan poisoning of SKF 525A and two other antagonists (iproniazid and 3-acetylpyridine) and their ability to antagonize barbiturate detoxification. This suggests that there is a common step in schradan activation and barbiturate detoxification, a possibility that is discussed more fully in Chapter 4.

Synergism ("Potentiation")

In 1956, it was reported that F.D.A. scientists had found certain combinations of organophosphates to be more toxic than one would expect on the assumption of additive effects.[7] The pair of compounds mentioned in this first anonymous article was malathion and EPN, used in feeding trials with dogs; in all the subsequent work, this particular pair of compounds, utilized with this particular animal, has consistently shown the most striking effects.

In 1957, the F.D.A. (United States Food and Drug Administration) workers Frawley *et al.*[75] reported that in acute oral studies on the rat, 50% were killed by about 1/10 LD_{50} each of malathion and EPN in combination. In dogs, a still more dramatic effect was found: all were killed by less than 1/40 LD_{50} of malathion in combination with less than 1/50 LD_{50} of EPN. Subacute feeding studies showed a greater-than-additive effect of this pair of compounds upon blood cholinesterase. In the rat, 500 p.p.m. of malathion or 25 p.p.m. of EPN had no effect separately; together they lowered the total blood cholinesterases 21%. In the dog, 100 p.p.m. of malathion or 20 p.p.m. of EPN had little effect upon erythrocyte cholinesterase; together, they reduced erythrocyte cholinesterase by 68%.

These authors used the term "potentiation" to describe the more-than-additive effect. At this stage there was no indication of whether the mechanism involved the improvement of the action of one compound by the influence of another (as, for instance, is true for piperonyl butoxide, which definitely enhances the effect of DDT). It was quite possible that some joint action was involved, such as has been described above in the case of atropine and 2-PAM in their protective action. Consequently, the term "synergism" seems more appropriate; it is a less loaded term, and states precisely what is needed, i.e., that the agents act together to produce an effect greater than the sum of their individual actions.

Later work by Williams *et al.*[176] gave data for all possible paired combinations of EPN, malathion, Systox, parathion and methyl parathion. These were fed to dogs over a six week period. Two feeding levels were studied, the so-called "safe" level, e.g., 100 p.p.m. of malathion, 20 p.p.m. of EPN, 2 p.p.m. of Systox, and the official U.S.A. "tolerance" level, e.g., 8, 3 and 0.75 p.p.m. for these three compounds. Only in the case of "safe" levels of Systox plus EPN was any synergism found with respect to plasma cholinesterase; in this case, 56% inhibition was found rather than the 44% predicted from simple additive effects. With respect to erythrocyte cholinesterase, only "safe" levels of malathion plus EPN showed synergism:

34% inhibition rather than the predicted 10%. Thus, no synergism was found at any "tolerance" level, so we may take it that where the standard legal requirements are met, synergism presents no consumer hazard with these compounds.

Data for synergism in the case of acute oral doses for mice were given by Rosenberg and Coon.[159] Using EPN, malathion, dimefox, and Phostex some synergism was found with all paired combinations. The term was used for cases where a combination of doses of the two compounds gave greater mortality than a double dose of either. Once again the most marked effect was found with EPN and malathion. Pairs of compounds that showed no synergism were: schradan with malathion, EPN, or parathion; malathion with parathion or eserine; and EPN with parathion.

Data for synergism in female rats were reported by DuBois[64] and are shown in Table 6.8. Only 3 out of 15 combinations tested were synergistic. In 6 cases mild antagonism was noted, and in 6 other cases the results were simply additive (evidence that this is an effective approach for establishing synergism). Murphy *et al.*[132] reported that TOCP (triorthocresyl phosphate) was an excellent synergist for malathion: 1/44 of an LD_{50} of TOCP

TABLE 6.8. COMBINATIONS OF ORGANOPHOSPHATES AGAINST FEMALE RATS[a]

Combinations	Per cent mortality
Additive	
Parathion + EPN	45
Parathion + Dipterex	55
Parathion + Systox	50
Systox + EPN	45
Systox + malathion	60
Guthion + EPN	60
Antagonistic	
Parathion + malathion	10
Parathion + Guthion	10
Dipterex + EPN	30
Dipterex + Systox	10
Malathion + Guthion	10
Systox + Guthion	5
Synergistic	
Malathion + EPN	100
Malathion + Dipterex	100
Guthion + Dipterex	100

[a] One-half LD_{50} of each compound administered simultaneously by intraperitoneal injection. The expected mortality, if the effects are simply additive, is 50%. Data of DuBois.[64]

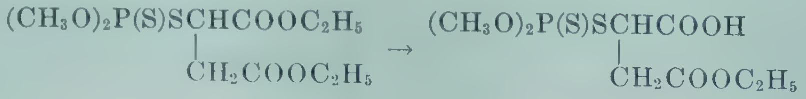
reduced the LD_{50} of malathion for rats from 1,100 to 61 mg./kg., if both compounds were given simultaneously. If the TOCP was given 24 hours before the malathion, the LD_{50} of malathion was reduced to 8 mg./kg.

The first demonstration of the mechanism of synergism came from Cook *et al.*,[37, 38] in 1957. They first showed, chromatographically, that malathion (alone among 12 organophosphates tested) was vigorously degraded by liver homogenates, and that this degradation was inhibited partially by Thimet, Systox, Phosdrin, Diazinon, and methyl parathion, and completely by parathion.

Manometric and colorimetric techniques were then used: they showed that malathion was hydrolyzed by liver homogenates, and that the hydrolysis was inhibited by this same group of organophosphates. The authors tentatively christened the hydrolyzing enzyme "malathionase," until its normal substrate should be known. In the case of phosphorothionates, greater inhibition of malathionase was obtained with the corresponding oxygen analog (produced by bromine oxidation) than with the phosphorothionate itself. The oxygen analogs gave inhibitions similar to other P=O compounds such as Phosdrin and Dipterex.

These results offer a thoroughly convincing explanation of why EPN synergizes malathion toxicity, i.e., malathion by itself is almost nontoxic because of its rapid degradation by malathionase of the liver. EPN inhibits malathionase, and thus greatly increases the effectiveness of malathion. Why is EPN so outstanding as a synergizer, since its oxidized form is little better than many other phosphates as an inhibitor of malathionase *in vitro*? The authors offer a very reasonable suggestion[38] using parathion as an example of a poor synergizer: "Parathion is so much more potent as an anticholinesterase agent than as an antimalathionase agent that its *in vivo* toxicity, due to its anticholinesterase action, is manifested before its toxicity due to its antimalathionase action is revealed and becomes great enough to render malathionase incapable of detoxifying malathion. EPN is considerably less effective as an anticholinesterase agent than parathion, but at the same time is equally effective as an antimalathionase agent." Unfortunately, no data is offered to support this explanation. One must bear in mind that what matters is the anticholinesterase activity either (*a*) of parathion as compared with EPN *in vivo* or (*b*) of paraoxon as compared with oxidized EPN *in vitro*.

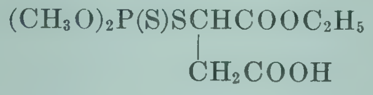
Cook and Yip[39] next studied the locus of action of malathionase. They concluded that a single carboxyester group of malathion was hydrolyzed, yielding what we might call "malathion acid":



$$(CH_3O)_2P(S)S\underset{\begin{array}{l}|\\CH_2COOC_2H_5\end{array}}{C}HCOOC_2H_5 \rightarrow (CH_3O)_2P(S)S\underset{\begin{array}{l}|\\CH_2COOC_2H_5\end{array}}{C}HCOOH$$

They did not, however, specify that it was the α-acid, as written here. Their evidence was as follows: (*a*) of all the organophosphates studied, only malathion was vigorously degraded by liver, and only malathion contained the carboxyester grouping; (*b*) identity of the synthetic monoacid with the crude product from the action of a liver acetone powder upon malathion, using a series of paper chromatographic systems; (*c*) identity of the synthetic monoacid with the chromatographically purified acetone-powder product, using infrared analysis; and (*d*) manometric data (Table 6.9) which they considered to indicate that only one ester link was hydrolyzed per molecule of malathion.

The results of Cook and Yip offer convincing evidence that malathion monoacid is produced when liver acetone powder acts upon malathion. Naturally, one cannot decide whether it is in fact the α-acid illustrated above, or the β-acid



$$(CH_3O)_2P(S)S\underset{\substack{|\\ CH_2COOH}}{C}HCOOC_2H_5$$

because the synthetic procedure (condensation of *O*,*O*-dimethyl phosphorodithioate with ethyl hydrogen maleate) could give either the α or β form. However, it must be pointed out that no proof was offered that the acetone powder gave the same product as (say) liver slices or homogenates. Also, the acetone powder-malathion incubate was extracted with chloroform; our own studies (below) show the dicarboxylic acid derivative (which we shall call malathion diacid) to be difficult to extract by chloroform under any conditions. Even the monoacid has a p*K* of about 3.7, and therefore would be more than 99.9% ionized at pH 7 and difficult to extract (Cook and Yip

TABLE 6.9. COMPARISON BETWEEN MALATHION ADDED AND MALATHION CALCULATED FROM CO_2 LIBERATED BY MALATHIONASE ACTION[a]

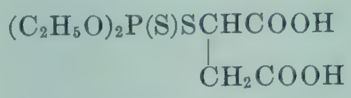
Malathion added (mg.)	CO_2 liberated[b] (μl.)	Recovery[c] (%)
0.3	24.9	123
0.6	42.7	105
0.9	61.6	101
1.2	78.6	97
1.5	89.1	87
1.8	101.4	83
2.4	120.0	74
3.0	121.7	60

[a] Data of Cook and Yip;[39] probably liver acetone powder was the malathionase source.

[b] Averages of data given; CO_2 is that which is liberated in 35 minutes.

[c] Based upon the assumption that 1 mole of malathion yields 1 mole of CO_2.

did not acidify their incubate prior to extraction). In short, there is nothing in this work to indicate whether the monoacid is the only or even the major metabolite.



$$(C_2H_5O)_2P(S)SCH(CH_2COOH)COOH$$

Malathion diacid

The manometric data given in Table 6.9 in fact suggest more than one hydrolysis per molecule. If one plots from the data of the table: "malathion added" against "per cent recovery," a linear relationship is found, suggesting that smaller malathion additions would have given even higher yields, i.e., well in excess of one hydrolysis per molecule. No data was offered to suggest that CO_2 evolution was complete in the 35 minute period of measurement. To be consistent with these authors' hypothesis, the first figure in the table would have to be 23% in error. The table therefore constitutes fairly good evidence that more than one mole of CO_2 was produced per mole of malathion hydrolyzed; there must be a second place of cleavage in the molecule. This aspect is discussed in more detail below.

Murphy and DuBois[133, 134] showed that the hydrolysis of malaoxon by rat liver and serum *in vitro* was markedly inhibited by feeding EPN to the animals or by injecting intraperitoneally EPN, Dipterex, or Guthion one hour before sacrifice. Feeding parathion, Guthion, or Dipterex had no effect on hydrolyzing activity. In the feeding study, 100 p.p.m. of EPN in the diet for two weeks inhibited the malaoxon-hydrolyzing ability 95% in liver and serum; with injection, 1.5 mg./kg. of EPN inhibited hydrolysis 75% in the case of liver and 100% in the case of serum. Later studies on injected TOCP showed that it too inhibited malaoxon hydrolysis by liver and serum. These inhibitions may be large enough in themselves to account for the striking effect of EPN and TOCP upon the toxicity of malathion. (The authors state that they are studying "the malathion detoxifying enzyme," but in fact their method examines malaoxon degradation.)

The effect of EPN upon malathion degradation by eleven rat tissues was studied by Seume and O'Brien,[166] using tracer-labeled malathion. They found that all of the eleven tissues studied degraded malathion rapidly and to a similar extent, the liver being ninth in order of activity (Table 6.10). In all tissues except ileum and brain, EPN (injected at the high level of 50 mg./kg., five hours before sacrifice) inhibited this degradation; but liver was ninth in order of sensitivity to inhibition. Malathion hydrolysis by liver was also inhibited by *in vitro* EPN, to an extent varying linearly with EPN concentration.

The nature of the degradation products in liver preparations *in vitro* was next examined, using an ion-exchange column with a series of solvents of increasing acidity (see Chapter 10). Ten water-soluble metabolites were found, of which the principal ones were malathion monoacid and diacid, which represented about 35% and 50% respectively of these metabolites. The relative amounts of these two acids varied with time, as one might expect, with prolonged incubation leading to a larger fraction of the diacid. Rats were then injected with EPN, the livers were removed after various times, and a study was made of the distribution of metabolites produced by the *in vitro* degradation of malathion. A pronounced shift of the pattern from the normal was found; thus, at its most extreme (5 hours after injection), the monoacid and diacid constituted only 15% instead of the normal 85% of the total products. Probably the fact that the liver hydrolysis was not as thoroughly inhibited as that of most tissues was due to its considerable phosphatase activity, which became very important when the carboxyesterase activity was inhibited by EPN.

One would expect that this inhibition of degradation would lead to increased tissue malathion levels and thence to increased malaoxon levels. However, it was found in all tissues studied (liver, blood, kidney, testis, and brain) that treatment by EPN *in vivo* actually severely decreased the production of malaoxon from malathion *in vitro*, presumably by competition with a phosphorothionate oxidizing system. The over-all result was a net decrease caused by EPN in the malaoxon level of these tissues in spite of a greatly increased malathion level.

The modifications produced by EPN in the metabolism of labeled malathion *in vivo* were studied in the rat and dog by Knaak and O'Brien.[104] The pattern of degradation was shifted, as above, from primarily carboxyes-

TABLE 6.10. MALATHION DEGRADATION IN VARIOUS TISSUES AND ITS INHIBITION BY EPN

Tissue	% Hydrolyzed after 10 minutes	% Inhibition by EPN[a]
Plasma	45	52
Kidney	44	85
Heart	41	23
Ileum	40	0
Testis	37	41
Spleen	34	38
Lung	31	86
Muscle	24	60
Erythrocytes	23	62
Liver	23	14
Brain	20	0

[a] Injected at 40 mg./kg., intraperitoneally, 5 hours before. Data of Seume and O'Brien.[166]

terase products to primarily phosphatase products. Malaoxon was detected only in rat blood, and its level was (as in the *in vitro* work) depressed by EPN. The level of malathion in rat blood and in the whole rat was substantially increased by EPN. Oddly enough, total urinary excretion of malathion products was affected very little by EPN in the rat or dog, an unexpected finding which could only be resolved by assuming that the phosphatase products are less rapidly excreted than the carboxyesterase products; probably the phosphatase products are also more readily degraded to orthophosphate and "lost" in the metabolic pool (cf. page 226).

Just what do we know about the mechanism of potentiation in this much-studied example of malathion with EPN? It seems clear that the first effect is blockage of the degradation of malathion and probably of malaoxon. But this does not appear to lead to increased tissue levels of malaoxon, as one would have expected. Instead, the malaoxon levels appear to be depressed due to an inhibition of oxidation. But if malathion's toxicity is due to a formation of malaoxon, how can EPN cause both a decrease in malaoxon and an increase in toxicity? Two possibilities exist: the malaoxon at some local crucial site may, in fact, be raised by EPN in spite of the generally decreased malaoxon level. This seems unlikely. Alternatively, the EPN may cause a persistence of tissue levels of malathion and hence a more persistent production of malaoxon, although at a low level. This persistence could produce an "aged" cholinesterase as has been described for other naturally and artificially persistent organosphosphates (page 183).

So much for mechanism. Seume and O'Brien[167] pointed out that if carboxyesterase inhibition is indeed the first step, then it should follow that such compounds as EPN and TOCP should also synergize any organophosphate having (like malathion) a low toxicity caused by its rapid degradation in the body by carboxyester hydrolysis. This proved to be the case: acethion and methyl methprothion were both synergized against mice by EPN and by TOCP. Furthermore, compounds which owe their low toxicity to carboxyamide hydrolysis were also synergized; these were dimethoate, CL18706, and acethion amide. It seems then that EPN and TOCP may inhibit carboxyamidases. Unfortunately for this delightfully reasonable set of observations, Dowco 109 was also synergized, yet it contains no carboxyester or carboxyamide grouping! At the moment, nothing is known of the metabolism of Dowco 109, so we cannot speculate as to whether EPN or TOCP could inhibit its degradation.

The question of terminology can now be discussed more fruitfully. The work on mechanism has shown beyond a doubt that with EPN plus malathion the EPN affects the toxicity of malathion and not *vice versa*. In this case then the term *potentiation* turns out to be appropriate, and one may speak of EPN as a *potentiator*, and perhaps of malathion as a *potentiated* *compound* (potentiatee??). In other cases where mechanism is uncertain, the term *synergism* should be used: it is quite possible that in some cases the effects of one upon the other are mutual: one then has no potentiator. Since *synergism* is a wider term, which embraces *potentiation*, it is the writer's opinion that terminology would be simplified by using it in all cases of greater-than-additive toxicity.

Metabolism and Residues in Intact Animals

In Chapter 4, the metabolism of organophosphates by various mammalian preparations was described. Such studies should be only preludes to the more important question: what happens to an organophosphate when administered *in vivo*? In fact, this question has received remarkably little attention, simply because it poses severe technical problems—unless we consider the extensive data on cholinesterase inhibition *in vivo* as an index of oxidation in the case of phosphorothionates and phosphoramidates. Effective studies with the whole mammal can probably be carried out only with tracer-labeled compounds, along with some effective separative technique. The most informative of such studies have come from Casida's group, using a variety of column chromatograms.

Hitherto in this book, an attempt has been made to write of groups or classes of compounds, partly to show that the organophosphates do represent a field in which useful generalities and predictions can be made, and partly to avoid the dreary catalogue appearance which results when one describes one compound after another. The reader is now asked to submit for a few pages to some compound-by-compound data, as the results are simply not extensive enough as yet to be presented in a more general way.

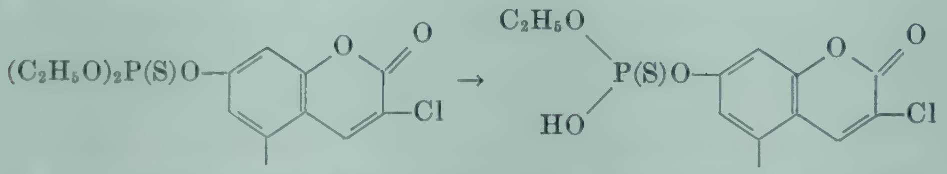
In these studies, the terms "chloroform-soluble metabolites" and "water-soluble metabolites" are often encountered. They describe those products which, respectively, can and cannot be extracted by chloroform from an aqueous system. Fortunately, the parent compound and its oxidation product are usually extractable by chloroform, and these are the compounds with actual or potential toxic effects; whereas the hydrolysis products usually are not extractable by chloroform, and have little toxic effect (but see page 61). With labeled compounds it is, of course, a particularly simple matter to extract the aqueous tissue preparation with solvent and then count the radioactivity in the two phases.

The terms "activating reaction" and "degrading reaction" will also be used. The first implies conversion to a more active anticholinesterase of moderate or good stability, and the second implies conversion to compounds which have no anticholinesterase activity, or else are so unstable as to be very poor inhibitors of cholinesterase under physiological conditions.

Co-ral

Krueger *et al.*[113] studied the metabolism and residues after administering labeled Co-ral to rats, a goat, and a cow. The studies were bedeviled by the phenomenon of very poor extractability of the insecticide from tissues by chloroform; similar difficulties were encountered with test extractions from other protein preparations, e.g., crystalline bovine albumen and milk. The extractability in these tests declined with time over several hours, eventually being reduced to as little as 20 % of the added amount, and in no case exceeding 60 %. A protein-binding of the Co-ral seemed possible. (However, MacDougall[120] finds that with an extraction technique, using acetone and benzene, more than 90 % can be recovered from all tissues studied.)

In the rat, the extent and nature of the excretion products varied considerably with the route of administration (Table 6.11). For instance, only with oral administration was much desethyl Co-ral formed:



Oral administration also gave more rapid excretion and proportionately more fecal as opposed to urinary excretion. About 50 % of the dose was recovered in urine 4 weeks after oral application; only 25 % following dermal application.

Table 6.11. Effects of Route of Administration upon Excretion of Co-ral in the Rat[a]

	Oral	Subcutaneous	Dermal
Per cent of dose excreted in urine and feces after 4 days	45	28	10
Per cent of dose excreted in urine and feces after 14 days	52	45	20
Per cent of urinary excretion products as *O,O*-diethyl phosphorothioate	65	75	33
Per cent as diethyl phosphate	10	20	33
Per cent as phosphoric acid	10	0	33
Per cent as desethyl Co-ral	15	5	0
Per cent of total excreted material in feces after 14 days	33	20	20
Per cent of fecal excretion products as Co-ral or its oxygen analog	55	17	55

[a] Figures are roughly averaged from data of Krueger *et al.*[113] Collection times from 72–96 hours after treatment. Doses: oral 50, subcutaneous 40, and dermal 45 mg./kg.

Excretion by the rat was also studied by Lindquist *et al.*[116] Using 20 mg./kg. orally, i.e., less than half the dose employed by Krueger *et al.*, they found 78% to be excreted in the urine within 24 hours; Krueger *et al.* found only 40% excreted in urine plus feces in that time. The discrepancy is rather alarming, especially since both used male white rats about 200-250 gm. body weight; both used corn oil as vehicle.

For the cow and goat, Krueger *et al.* used dermal application (the common route for Co-ral in practice, since oral administration is ineffective for cattle grub control). Extensive residue data on 42 tissues from a cow, 8 weeks after a dose of 40 mg./kg., showed surprisingly high levels of total residues in bone (10 p.p.m.); apart from the liver (1.2 p.p.m.) all other residues were less than 1 p.p.m. With the goat 6 days after a dose of 30 mg./kg., the liver had the highest residue (3.7 p.p.m.), bone was very low (e.g., 0.2 p.p.m.), and all others had less than 1 p.p.m. Probably the bone residue represents a sink into which material is incorporated following total degradation to phosphoric acid.

Residues in milk were of practical interest: in the cow a sharp maximum of 2 p.p.m. was found at 1 day with respect to Co-ral plus the oxygen analog (these two were not resolved), and a blunt maximum of 1.3 p.p.m. between 1 and 2 weeks for the ionic products. But in the goat no peak of Co-ral plus the oxygen analog was found, and the level never rose above 0.03 p.p.m., whereas the ionic products reached a peak of only 0.3 p.p.m. in 1 day and rapidly declined. There is therefore a striking difference, the cow giving significant and the goat negligible milk residues.

In both the goat and cow, analysis of the urinary products showed substantially the same pattern as in the rat, except that about half as much was as *O,O*-diethyl phosphorothioate, and about twice as much was as ethyl phosphate and phosphoric acid.

Results on cholinesterase values in the cow suggested that some activation occurred, producing the oxygen analog. This was not so in the goat. No more direct evidence about the occurrence of the oxygen analog was available, except that in one fecal sample from the cow (at 6 days) 6% of the radioactivity was due to the oxygen analog, compared with 32% as Co-ral.

The metabolism of Co-ral after spraying onto cattle was later studied by Robbins *et al.*,[156] using a dose similar to that of Krueger *et al.* By far the largest residue was found two weeks later on the hide of the animal; as a corollary, only 2–6% of the dose was recovered in the urine (Krueger *et al.* recovered about 15% of their dose in the urine of the goat). Robbins *et al.* also showed fairly substantial incorporations of P^{32} into phospholipids and phosphoproteins of the liver and kidney, presumably derived from orthophosphate obtained by degradation of the Co-ral. The highest residue of unaltered Co-ral, two weeks after treatment, was 1 p.p.m. in the subcutaneous fat.

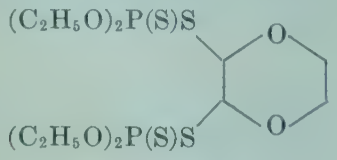
Diazinon

Robbins *et al.*[158] fed 20 mg./kg. of labeled diazinon to a cow. The blood level showed a peak of 3.2 p.p.m. at 12 hours, and declined rapidly to 0.4 p.p.m. in 48 hours. Of this blood residue, about 5% was hexane-soluble, and of this about 18% was Diazinon. The remainder chromatographed on paper as a single unidentified spot. The water-soluble fraction was probably made up of diethyl phosphate and *O,O*-diethyl phosphorothioate (however, the fraction does not seem to have been tested for other intermediates). In milk, the residues followed a similar peak (2.3 p.p.m. at 18 hours); these residues only constituted about 0.16% of the administered dose. About 50% of these residues was Diazinon.

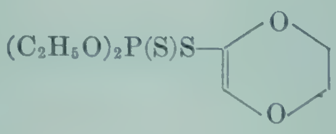
In urine, a blunter peak was found at about 6 hours (1 p.p.m.). In 36 hours, 74% was excreted by this route. The composition was about 45% diethyl phosphate and about 50% *O,O*-diethyl phosphorothioate. In the feces, a still broader peak at 24 hours was found (30 p.p.m.); in 36 hours only 7% was excreted by this route. The composition was not determined.

Delnav

This insecticide presents more than the usual number of problems because it is a mixture of at least eleven components. Arthur and Casida[9] found the following composition for technical Delnav, using chromatography on celite with acetonitrile-heptane:



(III), (IV) (*trans* and *cis*)



(II) Dioxene derivative

$(C_2H_5O)_2P(S)S\ S\ P\ (S)(OC_2H_5)_2$

(I*a*)

Besides these four identified components, the following were found: (I*b* and *c*), two unknown phosphorothioates, (V*a*, *b*, *c*, and *d*), four unknown phosphorus compounds, and (VI), unknown. The Roman numerals indicate the order in which the fractions were eluted from the celite column. Components (III) and (IV) were the principal ones, accounting for 68% by weight.

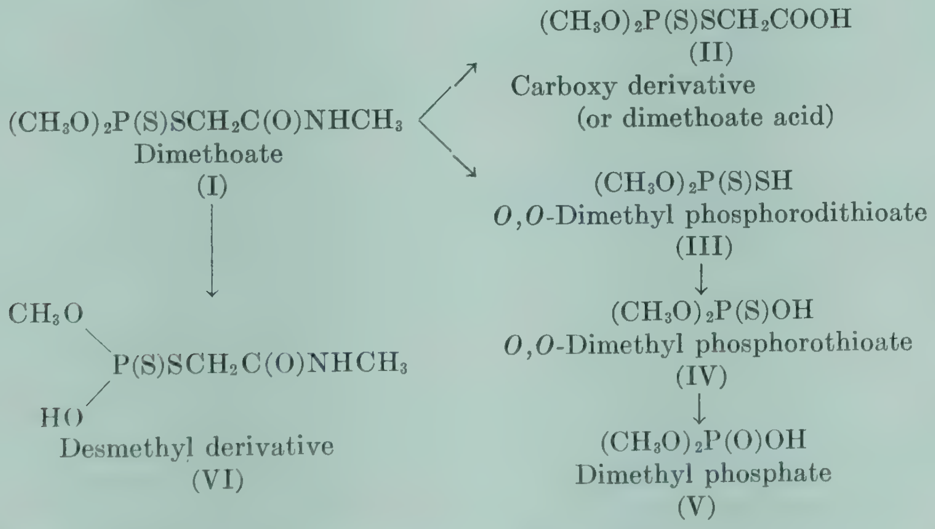
The metabolism of this mixture was studied in female rats[9] following oral treatment with radioactive Delnav. The rats were given 1 or 5 mg./kg. per day for 10 days. After the first day, they excreted about 45% of the dose in the urine each day, and a further 20% in the feces. Other studies involved a single oral dose of (III) or (IV) (15 mg./kg.). Analysis of the urine for both showed that about 80% of the phosphorus was as hydrolysis products, of which in turn 83% was as *O,O*-diethyl phosphorothioate, 6% as *O,O*-diethyl phosphorodithioate, and 11% as diethyl phosphate.

Residue studies were carried out 48 hours after treating female rats orally with 25 mg./kg. With (III), residues were highest in liver (2 p.p.m.), less in fat and kidney (1 p.p.m.), and below 1 p.p.m. in muscle, brain, and heart. With (IV), liver had 4 p.p.m., kidney 1 p.p.m., and the other tissues less than 1 p.p.m. Except for the fat, the residues of (III) and (IV) were usually over 90% water-soluble; in the fat, more than one-half was hexane-soluble.

DIMETHOATE

The metabolism and residues in rats, cows, and steers after oral treatment with dimethoate was studied by Dauterman *et al.*[45]

In the rat treated with 100 mg./kg. of dimethoate urinary excretion was extensive, but varied with sex: by two days urinary excretion was almost complete at 90% of the administered dose for males, but was only 55% for females. Fecal excretion totaled about 3% for males and 10% for females. Seven hydrolysis products were found in the urine, of which five were identified (II–VI).



$(CH_3O)_2P(S)SCH_2C(O)NHCH_3$
Dimethoate
(I)

$(CH_3O)_2P(S)SCH_2COOH$
(II)
Carboxy derivative
(or dimethoate acid)

$(CH_3O)_2P(S)SH$
O,O-Dimethyl phosphorodithioate
(III)

$(CH_3O)_2P(S)OH$
O,O-Dimethyl phosphorothioate
(IV)

$(CH_3O)_2P(O)OH$
Dimethyl phosphate
(V)

$(CH_3O)(HO)P(S)SCH_2C(O)NHCH_3$
Desmethyl derivative
(VI)

The scheme by no means necessarily shows the actual routes followed. For instance, the data on cholinesterase inhibition showed that there was undoubtedly some oxidation of dimethoate to its oxygen analog (P=S → P=O) and subsequent hydrolysis could lead to (IV) and to (V), if both the P—S and S—C bonds were cleaved. Similarly, hydrolysis of (II) or (VI) would also yield (III) or (IV). Table 6.12 shows the relative amounts of these metabolites in the rat and steer at different times. In both animals, dimethoate acid is at first predominant, accounting for 74% of the products for the steer. After a day, the picture in the rat is little changed except for a 10% increase in dimethyl phosphate at the expense of the desmethyl; but in the steer the fraction of dimethoate acid has dropped 56%, most of which is balanced by a 42% increase in dimethyl phosphate. Subsidiary evidence agreed with the above data in pointing to an amidase action (to produce dimethoate acid) as the major initial degradation in both species.

The total residues in milk, blood, and body fat were examined at various times in cows which had been fed 10 mg./kg. of dimethoate. In blood and subcutaneous fat, a peak at 4 hours was seen, with 6 p.p.m. in the blood (of which about 30% was chloroform-soluble) and 0.8 p.p.m. in the fat. By two days both levels were below 0.1 p.p.m. In milk, on the other hand, a steady slow decline was seen, from an initial 1.5 p.p.m. to 0.3 p.p.m. after 6 days. But a marked change in the composition of the milk residues occurred: at 12 hours about 40% was chloroform-soluble; at 6 days this had dropped to 4%.

Extensive data were given for tissue residues 12 days after treatment (34 tissues were examined). Recovery tests showed poor extractions when dimethoate was added to tissues, ranging from 52% recovery from the brain to 74% from fat. The total residues (presumably uncorrected for this partial recovery) after 12 days were in all cases extremely small: highest was liver at 2 p.p.m., next parotid gland at 0.5 p.p.m., the remainder fell mainly between 0.02 and 0.3 p.p.m. These levels are substantially less than those for Co-ral or ronnel, the other important animal systemics.

In no tissue was any oxygen analog of dimethoate found, although the

TABLE 6.12. METABOLITES IN URINE AFTER TREATMENT OF RATS AND STEERS WITH DIMETHOATE[a]

Metabolite	Percentage of total urinary metabolites			
	Rat (male)		Steer	
	2 hours	24 hours	1 hour	30 hours
(II) Dimethoate acid	32	35	74	18
(III) *O,O*-Dimethyl hydrogen phosphorodithioate	23	27	12	17
(IV) *O,O*-Dimethyl phosphorodithioate	21	17	9	13
(V) Dimethyl phosphate	6	18	2	46
Unknowns (two)	6	2	1	2
(VI) Desmethyl derivative	12	2	2	4

[a] Data from Dauterman *et al.*,[48] rounded off to whole numbers. Roman numerals refer to text.

cholinesterase depressions showed some had formed. Very probably, there is a steady production of the analog which then phosphorylates cholinesterase (and other enzymes and proteins), and therefore never reaches substantial levels. A large fraction of the chloroform-soluble metabolites were, however, present as some other compound(s) than dimethoate, e.g., 90% in the feces, liver, and kidney at 12 days, 80% in the milk at 10 days. This very large unknown component is not the oxygen analog nor is it *O*,*O*,*S*-trimethyl phosphorodithioate or phosphorothioate.[44] These latter could, conceivably, have been derived from decarboxylation of the dimethoate acid:

$$(CH_3O)_2P(S)SCH_2COOH \rightarrow (CH_3O)_2P(S)SCH_3 \rightarrow (CH_3O)_2P(O)SCH_3$$

The unknown fraction was eluted from a silica gel column by methanol; it could be one or more compounds.[44]

A later study of dimethoate metabolism in calves by Kaplanis *et al.*[97a] demonstrated the extensive degradation and excretion, but failed to identify the principal degradation product.

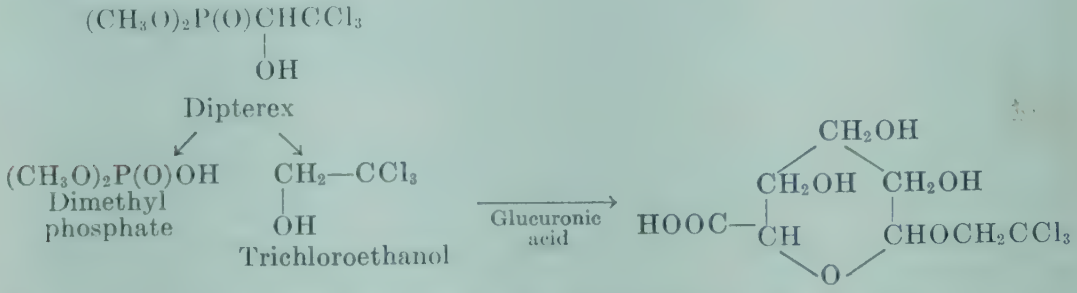
DIPTEREX

The metabolism of P^{32}-labeled Dipterex in the cow was studied by Robbins *et al.*,[157] following oral application of 25 mg./kg. A peak level of radioactivity was found in the blood in 2 hours equivalent to 15 p.p.m. of Dipterex, in the milk at 18 hours equivalent to 2.3 p.p.m., in the urine at 6 hours equivalent to 1.4 p.p.m., and in the feces at 18 hours equivalent to 55 p.p.m. Urinary excretion was the most important outlet, accounting for about 65% of the dose by 12 hours. Fecal output was very small, the total output over 4 days representing only 3% of the dose.

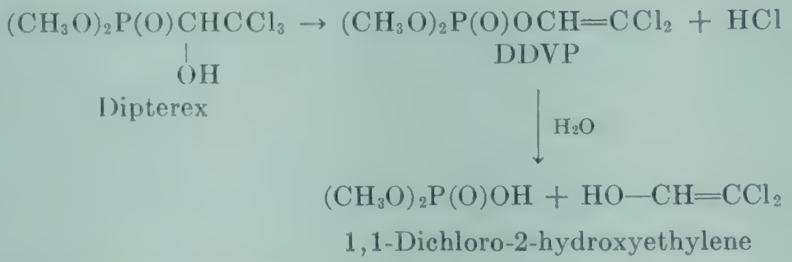
Of the blood radioactivity, only about 7% was ever present as Dipterex; the remainder was water-soluble. No trace of DDVP (a possible product of the dehydrochlorination and rearrangement of Dipterex) was found. The urine analysis showed 17% of dimethyl phosphate or phosphite, and 78% of an unidentified metabolite.

Arthur and Casida[8] studied Dipterex metabolism in the dog following intravenous injection of 150 mg./kg. of the labeled compound. Both degradation and excretion were astonishingly rapid: the theoretical original blood level of 1780 p.p.m. of Dipterex itself fell in 9 minutes to 85 p.p.m., and in 6 hours to 8 p.p.m. Hydrolysis products in the blood accounted for a part of this loss: in 9 minutes they had reached 159 p.p.m., at 30 minutes they reached their maximum of 167 p.p.m., and then the level fell steadily to reach 8 p.p.m. in 24 hours. Enormous levels occurred rapidly in the urine: the 0–6 hours sample had 2060 p.p.m. of Dipterex and 7300 p.p.m. of hydrolysis products. The next urine sample, 6–24 hours, contained only 36 p.p.m. of Dipterex and 106 p.p.m. of hydrolytic products; by 48 hours, excretion was virtually complete.

The fate of the nonphosphorus moiety of the hydrolyzed Dipterex was examined; the evidence was in favor of it being trichloroethanol conjugated with glucuronic acid. If so, we may tentatively indicate the degradation of Dipterex in the dog:



There is no precedent for this direct hydrolysis of a P—C bond, and one has to consider the possibility that Dipterex is dehydrochlorinated and rearranged to give DDVP[124] (a well-established reaction *in vitro*, see page 67) which then hydrolyzes in the usual way:



$$\underset{\text{Dipterex}}{(CH_3O)_2P(O)\underset{\displaystyle OH}{\underset{|}{C}}HCCl_3} \rightarrow \underset{\text{DDVP}}{(CH_3O)_2P(O)OCH{=}CCl_2} + HCl$$

$$\xrightarrow{H_2O} (CH_3O)_2P(O)OH + \underset{\text{1,1-Dichloro-2-hydroxyethylene}}{HO{-}CH{=}CCl_2}$$

The evidence against this is that (*a*) DDVP was not found in the Dipterex-poisoned animal, and (*b*) when the potassium salt of the urinary glucuronide from the Dipterex-poisoned animal was hydrolyzed and then oxidized with dichromate, a material was obtained which had the same pentane-water and hexane-water partition coefficients and gave the same Fujiwara test as dichromate-oxidized trichloroethanol. This evidence is not conclusive, but should probably be tentatively accepted.

Metcalf *et al.*[126] take a view contrary to that of Arthur and Casida. They have evidence that in the housefly *in vivo* conversion of Dipterex to DDVP does occur: their strongest evidence is that they actually identified, chromatographically, some DDVP in Dipterex-treated flies. They felt that the anticholinesterase action of Dipterex, under physiological conditions, was due to DDVP production, and this argument would be applicable to the mammal as well as the insect. In support of this conclusion: (*a*) they cited Arthur and Casida's observation[8] that cholinesterase inhibited by DDVP or by Dipterex is the same, as judged by its recovery rate; (*b*) they showed that Dipterex dehydrochlorinates fairly readily to DDVP under mildly alkaline conditions (page 67); and (*c*) they showed that Dipterex inhibits cholinesterase *in vitro* poorly under acid conditions when dehydrochlorination is slow, well under alkaline conditions when dehydrochlorination is fast. They also claimed that "DDVP inhibits strongly at either acid or alkaline pH," but their supporting data shows 100% inhibition by DDVP at all pH's, so one can tell nothing of the pH-dependence of this inhibition.

Since the data of Metcalf *et al.* show an I_{50} at pH 6 of about 10^{-7} M, and the dehydrochlorination half-life is then 98 hours, one can hardly doubt that Dipterex is quite a potent cholinesterase "in its own right." Furthermore, at pH 7 (presumably the pH of importance *in vivo*) the dehydrochlorination half-life is 6.5 hours, and this seems a slow process to account for the effects of Dipterex, e.g., knockdown of houseflies in 48 seconds. In conclusion, it seems reasonable to assume that Dipterex has a direct effect on cholinesterase, and that its *in vivo* action is mainly attributable to this effect. Probably, DDVP production occurs to a small extent *in vivo*. However, if the sample of Dipterex is impure, or if it is exposed to alkaline conditions, its toxicity may well be caused entirely by its content of DDVP.

Malathion

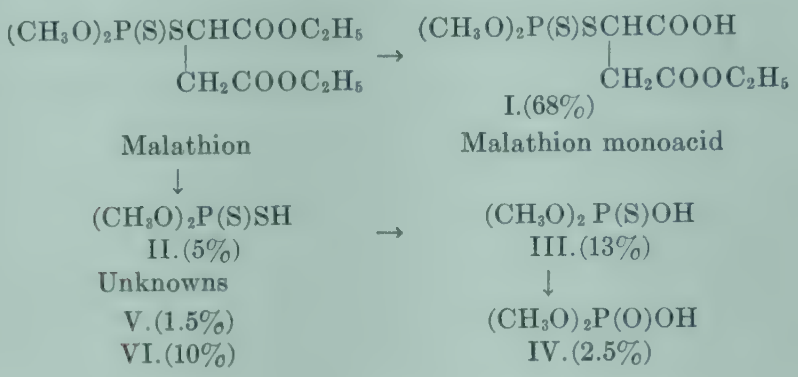
Metabolism and residues of malathion in the hen and mouse were studied by March *et al.*[121] using radioactive malathion and paper chromatography. On feeding hens a dose of 100 p.p.m. daily in the diet, about half of the daily dose appeared each day in the droppings. On stopping treatment, the levels fell within 3 days from the treatment level of 50 p.p.m. in the droppings to 2, and in 10 days to 0.3 p.p.m. The phosphorus of these residues was about 98% water-soluble, so degradation was clearly extensive. On this regimen, total body residues were studied; they rose to 2–3 p.p.m. in the liver, to about 1 p.p.m. in kidney, breast, and blood, and less in gizzard and drumstick. Ten days after stopping treatment, these levels had dropped to one-tenth of their treatment level. The residues were roughly 10% chloroform-soluble. In eggs layed by these birds, the total residue rose steadily to 0.1 p.p.m. and on stopping treatment, declined slowly to 0.04 p.p.m. in eleven days. Comparable data were also given for birds sprayed with malathion, and some figures for intraperitoneally injected birds.

Chromatographic data showed at least seven components of the water-soluble residues in the droppings. In the urine of the mouse injected intraperitoneally with 600 mg./kg., at least four components were seen. None of these was identified.

March *et al.*[122] reported on the malathion residues in calves sprayed twice (one week between sprays) with about 50 mg./kg. of malathion, and sacrificed either one or two weeks after the second application. Extensive total tissue residues were reported, and were typically about 0.1 p.p.m., except for liver which had about 1 p.p.m., and bone which had 1–2 p.p.m. No metabolites were identified, nor was the quantity excreted measured.

Gjullin *et al.*[78] observed that cows feeding on pastures sprayed with 0.4 lbs. of malathion per acre had no detectable malathion or off-flavor in their milk.

Malathion metabolism in the mouse was studied by Krueger and O'Brien[114] using column chromatography. This work will be described in detail in Chapter 9. Now we need only mention that 30 minutes after injecting 30 mg./kg. intraperitoneally, the water-soluble fraction present in the whole mouse was separated into the following six components:



$$\underset{\text{Malathion}}{(CH_3O)_2P(S)SCHCOOC_2H_5 \atop \quad\quad\quad\quad\quad | \atop \quad\quad\quad\quad\quad CH_2COOC_2H_5} \rightarrow \underset{\text{I.(68\%)} \atop \text{Malathion monoacid}}{(CH_3O)_2P(S)SCHCOOH \atop \quad\quad\quad\quad\quad | \atop \quad\quad\quad\quad\quad CH_2COOC_2H_5}$$

$$\begin{array}{ccc} \text{Malathion} & & \\ \downarrow & & \\ (CH_3O)_2P(S)SH & \rightarrow & (CH_3O)_2P(S)OH \\ \text{II.(5\%)} & & \text{III.(13\%)} \\ \text{Unknowns} & & \downarrow \\ \text{V.(1.5\%)} & & (CH_3O)_2P(O)OH \\ \text{VI.(10\%)} & & \text{IV.(2.5\%)} \end{array}$$

The figures in parentheses indicate the per cent of each in the water-soluble fraction. As before, the pathway shown is only a suggestion of one of those possible.

Using the same techniques, the metabolism of labeled malathion in the lactating cow was examined later by O'Brien *et al.*[145] An oral dose of 1.3 mg. per kg. was given on three successive days. The urine was the principal excretory route, accounting for 90% of the excreted material; feces accounted for 9.7% and milk for 0.3%. However, 23% of the dose was not excreted in the three week period studied, and was presumably incorporated into the large metabolic pool of phosphorus. The percentage lost in this way will, presumably, be greatest at small doses: thus, if the amount of orthophosphate produced from a labeled phosphorus insecticide is equal to the size of the body's phosphate pool, about one-half will be "lost" as far as excretion is concerned; but if it is ten times the pool, roughly one-tenth will be lost, and so on.

Blood levels were studied and showed a sharp peak of activity 1 hour after dosing when the blood contained an estimated 18% of the dose.

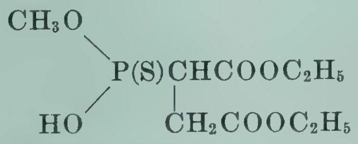
The nature of the urinary metabolites has been studied in the cow,[104] rat, and dog.[104] The results, shown in Table 6.13, show substantial variation with species: malathion monoacid represented a large proportion (63%) in the cow, 40% in the dog, and only 12% in the rat. The diacid proportion fell in the reverse order, being 17% in the cow, 21% in the dog, and 48% in the rat. Some of this variation may have been due to the fact that the cow received low doses (3 daily at 1.3 mg./kg.), whereas the rat and dog

TABLE 6.13. URINARY METABOLITES OF MALATHION IN COW, RAT, AND DOG[a]

Animal and dose	Urine used	Malathion monoacid	Malathion diacid	Desmethyl malathion	Dimethyl phosphorodithioate	Dimethyl phosphorothioate	Dimethyl phosphate
Cow (1.3 mg./kg. for 3 days)	1 week composite	63	17	7	Trace	11	2
Rat (25 mg./kg.)	48 hour composite	12	48	11	4	10	6
Dog (25 mg./kg.)	Average for 48 hours	40	21	21	7	7	1

[a] Results are per cent of solvent-unextractable metabolites of each compound. Data of Knaak and O'Brien,[104] and O'Brien *et al.*[145]

received a higher dose of 25 mg./kg. Desmethyl malathion represented a fairly substantial fraction, being as high as 21% of the solvent-inextractable metabolites in the dog urine.



Desmethyl malathion

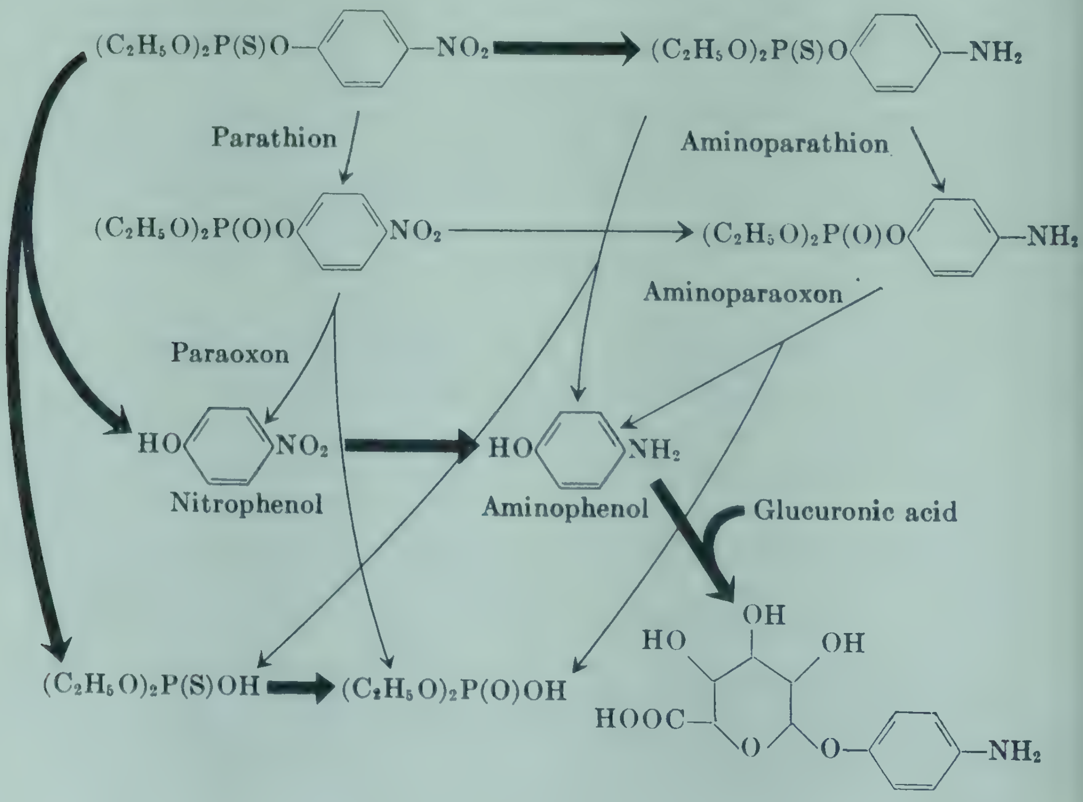
Fecal metabolites were studied in the cow, and a pattern quite unlike that in urine was found: dimethyl phosphate was the major metabolite (47%) and the monoacid and diacid totaled only 15%. This may indicate a different route for degradation by rumen microorganisms.

PARATHION

The metabolism of parathion in dairy cows has been studied by Dahm and co-workers.[41, 149] They observed that large intake levels (14 p.p.m. or 0.3 mg./kg./day) produced no symptoms, nor was parathion or *p*-nitrophenol found in milk, blood, or urine (the assay, which was that of Averell and Norris, would also have detected paraoxon, aminoparathion, aminoparaoxon, or aminophenol[87]). However, the normal level of diazotizable material (i.e., aromatic amines) in the urine was raised by the treatment, and increasing the parathion dose increased the level of diazotizable material. After vigorous acid hydrolysis, the urine gave a positive test for *p*-aminophenol. Furthermore, levels of glucuronic acid in the urine from treated cows were higher than those in normal cows. The authors concluded that the urinary excretion product was *p*-aminophenyl-glucuronide. The reductive step could precede or follow hydrolysis.

Ahmed *et al.*[2] used P^{32}-labeled parathion in the cow, administering two 7 mg./kg. oral doses with a five day interval. The parathion level in the rumen fell rapidly, most of it being converted to aminoparathion, and a part hydrolyzed (either before or after reduction) to *O*,*O*-diethyl phosphorothioate. Radioactivity was also found in blood, urine, and feces, with a trace in milk. Peak activities, expressed as parathion, were at 10 hours in urine (400 p.p.m.), 30 hours in feces (50 p.p.m.), 0.5 hours in rumen juice (8 p.p.m.), and 1 hour in blood (6 p.p.m.). Some of their data on the composition of these residues is given in Table 6.14. In the case of urine, further analysis of the hydrolysis products showed *O*,*O*-diethyl phosphorothioate principally (74%) and the rest as diethyl phosphate.

These data tend to confirm Dahm's findings of very small residues in the milk and blood. However, they show substantial aminoparathion levels in urine, which Dahm's Averell-Norris assay should have detected. The major fraction is as hydrolysis products, and these doubtless constitute the phosphorus moiety, whose other portion was recovered by Dahm as the glucuronide. The table also shows that very little combined nitrophenol escapes reduction; aminoparathion is a very substantial metabolite. Combining this and Dahm's study we may suggest the over-all scheme shown here (I) for the cow (the heavy arrows represent reasonable guesses at the most important metabolic pathways):


(C₂H₅O)₂P(S)O— —NO₂
(C₂H₅O)₂P(S)O —NH₂
Parathion
Aminoparathion
(C₂H₅O)₂P(O)O NO₂
(C₂H₅O)₂P(O)O —NH₂
Aminoparaoxon
Paraoxon
HO NO₂
Nitrophenol
HO NH₂
Aminophenol
Glucuronic acid
(C₂H₅O)₂P(S)OH
(C₂H₅O)₂P(O)OH
OH
HO
OH
HOOC—
O
O—
—NH₂

(I)

TABLE 6.14. METABOLISM OF PARATHION (7 MG./KG., ORAL) BY THE COW[a]

Compound	p.p.m. of metabolite in			
	Rumen juice 6 hours	Blood 6 hours	Milk 6 hours	Urine 0–24 hours
Parathion	0.7	0.07	0.04	1.2
Aminoparathion	2.6	0.04	0.11	170
Paraoxon	0	0.04	0.07	0
Aminoparaoxon	0	0.42	0.61	8
Hydrolysis products	1.6	2.7	0.79	239

[a] Data of Ahmed *et al.*[2]

For the male rat, Ahmed *et al.*[2] found a metabolic pattern quite unlike that for the cow. After 10 mg./kg. orally, 66% was excreted in the urine in 24 hours (compared with 43% in the cow after 5 days). This urinary output was 99.5% as hydrolysis products (compared with 57% in the cow). As we shall see below, aminophenol (bound or free) is not a major excretory product in the nonruminant.

The metabolism following administration of parathion intravenously to dogs was studied by Gardocki and Hazleton.[77] At a dosage of 10 mg./kg., about 2% was recovered from urine as *p*-aminophenol derivative(s), e.g., aminoparathion or aminoparaoxon. They then proceeded with assays which determined total *p*-aminophenol plus *p*-nitrophenol derivatives. This data, then, is for the nonphosphorus moiety. They recovered from urine 50% of this moiety in 24 hours, following dosage at 2 mg./kg., or 81% following 4 mg./kg.

The authors say that "the principal urinary excretion product . . . is probably *p*-nitrophenol." In fact, all their assays were preceded by hydrolysis, and did not differentiate between free or combined *p*-nitrophenol. The conclusion is that the principal urinary products either are or contain *p*-nitrophenol. There are four possibilities: the *p*-nitrophenol could occur free, as parathion, as paraoxon, or as a glucuronide. Evidently negligible reduction occurs in the dog.

Jensen *et al.*[97] used parathion labeled with S^{35} which was applied dermally to rabbits at 125 mg. per animal. The label appeared in urine and blood; in urine a maximum level of 10 p.p.m. was reached in 6 days, which fell to 2 p.p.m. in 14 days. In blood, the maximum level was at 4 days (0.8 p.p.m.). Tissue residues were examined after 5 and 20 days; at 5 days, none exceeded 0.5 p.p.m., but at 20 days blood showed 4 p.p.m. and spleen 2 p.p.m. Liver showed only 0.3 p.p.m. on both days. These results are expressed in terms of parathion. It is not easy to evaluate these results, since a great deal of the sulfur is almost certainly removed from the phosphorus by an early oxidation of parathion, and more by the oxidation of the *O*,*O*-

diethyl phosphorothioate which would result from parathion hydrolysis. Liver commonly shows higher residues than other tissues for most insecticides, but in this study had quite a low level. In short, these data should not be used as an index either of parathion or paraoxon.

Phosdrin

Casida *et al.*[33] conducted experiments on cows fed at various Phosdrin levels (up to 40 p.p.m.) for seven days or given a single oral dose (2 mg./kg.). Radioactive (P^{32}) Phosdrin was used.

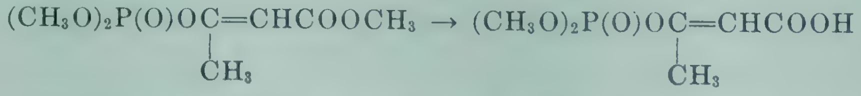
Residues in milk were extremely small in all cases. For example, in the cow given 2 mg./kg., the highest level was found at 6 hours, and was only 0.06 p.p.m. of unchanged Phosdrin. In the cow fed 40 p.p.m. in the diet, 0.06 p.p.m. was again the maximum milk level found. As for total residue levels (i.e., Phosdrin plus labeled metabolites), these never exceeded 0.3 p.p.m. Total residues for 29 tissues were given in the case of the seven day feeding study; the cow was killed after the seven day period. The highest level was in the liver (1.2 p.p.m.). All other tissues had less than 0.5 p.p.m.

For both 7-day feeding and single dosing, the principal excretory route was the urine, accounting for about 50%. Feces residues accounted for about 20% of the dose in the singly-dosed animals, and about 15% in those fed daily with Phosdrin. The feces products were all water-soluble, and, therefore, were all hydrolysis products. The urine products were specifically identified as entirely dimethyl phosphate.

Phosdrin therefore presents a charmingly simple metabolic picture. It is rapidly degraded by hydrolysis, and we may confidently write the overall reaction as far as phosphorus is concerned as:

$$\mathrm{(CH_3O)_2P(O)O\underset{\overset{|}{CH_3}}{C}{=}CHCOOCH_3} \rightarrow \mathrm{(CH_3O)_2P(O)OH}$$

Nevertheless, one cannot exclude the possibility of hydrolysis to Phosdrin acid occurring first

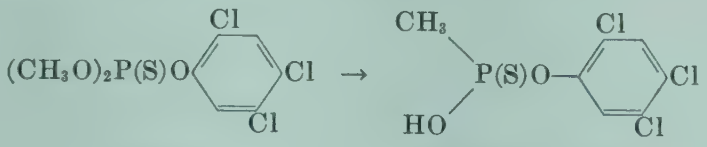


$$\mathrm{(CH_3O)_2P(O)O\underset{\overset{|}{CH_3}}{C}{=}CHCOOCH_3} \rightarrow \mathrm{(CH_3O)_2P(O)O\underset{\overset{|}{CH_3}}{C}{=}CHCOOH}$$

with subsequent hydrolysis to dimethyl phosphate. This is an unlikely possibility.

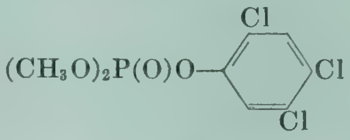
Ronnel (Viozene, Trolene, Dow ET14, Dow ET57)

Plapp and Casida[151] in 1958 studied the metabolism of ronnel in rats and a cow. In both cases, excretion was mainly in the urine (total in 5 days about 60% in the rat and 45% in the cow) and very little in the feces. Of very great importance was their observation, derived from ion-exchange chromatography, that in the rat about one-half of the urine product was the product of a hitherto undescribed *O*-alkyl split:



Ronnel   Desmethyl ronnel

The technique did not distinguish between the product pictured here and the corresponding phosphate. The remainder of the urine product was principally dimethyl phosphate, with less *O*,*O*-dimethyl phosphorothioate (it was of course impossible to determine whether the phosphate was formed by oxidation of the phosphorothioate, or by cleavage of oxidized ronnel). The phosphate analog of ronnel was also fed:

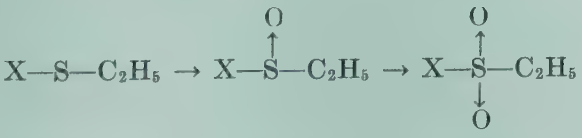


Again about one-half of the metabolites in the urine was formed by the *O*-alkyl split.

Extensive studies of total residues were made after feeding 100 mg./kg. to rats and a cow. In the rat, it was shown that the tissue residues rose to a maximum within about 0.5 days, then fell off rapidly. The maximum was 60 p.p.m. in liver, 40 in subcutaneous fat, and 10 in heart. In about 12 days the residues leveled out to around 5 p.p.m. in all tissues. In the cow, the residues 7 days after feeding 100 mg./kg. were studied in 19 tissues. The highest total was 80 p.p.m. in kidney; levels in fat were high (18–44 p.p.m.), and all the others fell between 6 and 33 p.p.m.

Systox

March *et al.*[123] have studied the metabolism of Systox in the white mouse. The principle activating reactions involve oxidations of the thioether sulfur, first to the sulfoxide, then to the sulfone:



$$X{-}S{-}C_2H_5 \rightarrow X{-}\overset{\overset{O}{\uparrow}}{S}{-}C_2H_5 \rightarrow X{-}\underset{\underset{O}{\downarrow}}{\overset{\overset{O}{\uparrow}}{S}}{-}C_2H_5$$

Thioether   Sulfoxide   Sulfone

where X is $(C_2H_5O)_2P(S)OC_2H_4$ for the thiono isomer or $(C_2H_5O)_2P(O)SC_2H_4$ for the thiol isomer. Another possible oxidation route would be of the thiono isomer to the corresponding phosphate:

$$(C_2H_5O)_2P(S)OC_2H_4SC_2H_5 \rightarrow (C_2H_5O)P(O)OC_2H_4SC_2H_5$$

Phosphorothionate Phosphate

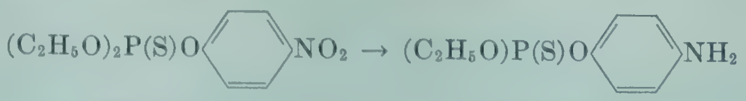
The phosphate should then be oxidizable to its sulfoxide and sulfone. Besides these intermediates, we may expect all their hydrolysis products, of which the phosphorus-containing parts should be *O,O*-diethyl phosphorothioate and/or diethyl phosphate. The observed facts were after feeding either isomer: rapid adsorption, very small excretion in feces (e.g., 1% in 24 hours), considerable excretion in urine (e.g., 74% in 24 hours) of which the major part (71%) was as unidentified water-soluble metabolites. A great deal of painstaking work was put into attempting to identify the products, but clearly the paper chromatographic techniques were inadequate. Thus, the following groups were not resolved:[76, 123] (*a*) phosphate sulfoxide, phosphate sulfone, and phosphorothionate sulfone; (*b*) phosphorothiolate sulfoxide and sulfone; and (*c*) *O,O*-diethyl phosphate and phosphorothioate. Also artifactitious double-peaking was noted in two areas of the chromatograms. By using anticholinesterase data, as well as R_f's,* the authors were able to identify positively the phosphorothionate sulfoxide and the phosphate.

In summary, the complexity of the oxidations and the inadequacy of the separative technique do not allow unambiguous conclusions as to the nature of the metabolites produced.

Influence of Rumen Microorganisms

On a strictly rational basis, this section should be included in Chapter 4, since it mainly describes *in vitro* effects. However, our primary interest in rumen microorganisms is to discover how they influence poisoning of ruminants, and this factor only arises with the intact animal.

Cook[36] studied the effects when 10 different organophosphates were incubated at 100 p.p.m. with fresh rumen juice (*a*) from cows on a high-alfalfa ration and (*b*) from cows on a high-grain ration. In general, all those compounds containing the *p*-nitrophenol group disappeared rapidly: parathion, paraoxon, Chlorthion, and EPN. Only minor loss occurred with malathion, Phosdrin, Dipterex, Diazinon, Delnav, and Systox. The "disappearance" referred to was judged by anticholinesterase assay, and could be produced by various means. In the case of parathion, Cook showed that the rumen juice reduced it at the nitro group.

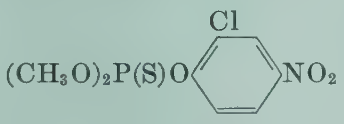


Parathion Aminoparathion

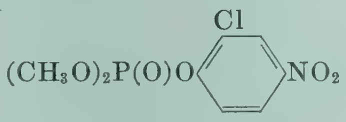
$$* R_f = \frac{\text{distance traveled on chromatogram by component}}{\text{distance traveled by solvent}}$$

Aminoparathion is a very poor anticholinesterase, as would be expected from the enormous difference in inductive and mesomeric effects between the nitro and amino substituents (page 389). Cook's evidence was (*a*) chromatographic identity of the rumen metabolite with the product of zinc-HCl reduction of parathion; (*b*) positive reaction of rumen metabolite with the Averell-Norris assay without prior reduction (parathion itself has to be reduced, first, to aminoparathion). There was evidence that some unidentified microorganism in rumen juice carried out the reduction.

Ahmed *et al.* carried out a more detailed study of several organophosphates with the results shown in Fig. 6.10. Here EPNO refers to the oxygen analog of EPN; other compounds are:



American Cyanamid 4124



American Cyanamid 4138

The figure shows that in five of the six compounds, hydrolysis was of more importance than reduction as a factor in degrading the parent compound.

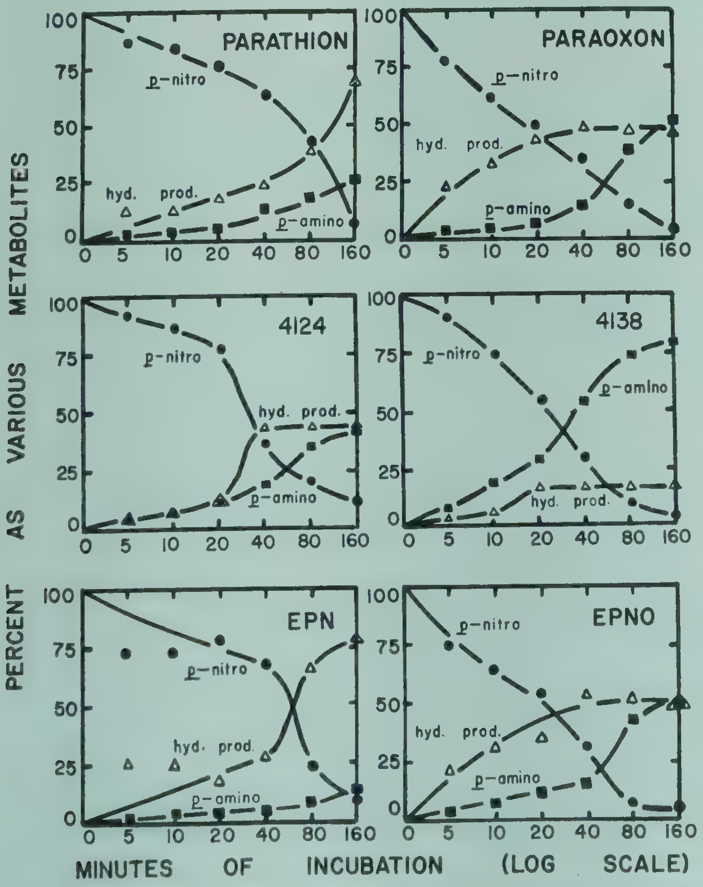


FIG. 6.10. Hydrolysis and reduction of organophosphates by stagnating bovine rumen juice. All compounds at 300 p.p.m. From Ahmed *et al.*[2]

TABLE 6.15. HYDROLYSIS OF ORGANOPHOSPHATES BY RUMEN JUICE[a]

Compound	% Hydrolyzed in 24 hours
(*a*) *Extensive hydrolysis*	
$(C_2H_5O)_2P(S)SP(S)(OC_2H_5)_2$	77
Malathion	76
TEPP	91
(*b*) *Moderate hydrolysis*	
Phosdrin	28
$(C_2H_5O)_2P(S)OC_2H_5$	54
$(C_2H_5O)_2P(S)SC_2H_5$	54
Sulfotepp	47
Thimet	56
Disyston	58
Systox	34
Delnav	60
Tetram	55
$(C_2H_5O)_2P(O)OCH{=}CHCl$	31
(*c*) *Little hydrolysis*	
Dimethoate	8
Ronnel	8
Schradan	7

[a] Data of Ahmed *et al.*[2] Not corrected for nonenzymic hydrolysis. All at 250 p.p.m. except malathion (50 p.p.m.) and ronnel (20 p.p.m.).

The hydrolysis of 16 other compounds was studied, with the results shown in Table 6.15. Clearly, hydrolysis was very extensive for malathion and TEPP, and of considerable importance for ten other compounds. Unfortunately, one cannot use these data directly for conclusions as to the role of microorganisms, as the figures include the contribution of nonenzymic hydrolysis.

If there were more LD$_{50}$'s available for organophosphates against ruminants, the interesting information described above could be applied to explain unusually low susceptibilities. For economic reasons, such data are not likely to be available. However, it is well-established for parathion that very high levels (e.g., feeding up to 2,000 p.p.m., or 32 mg./kg./day) given to the cow produce negligible cholinesterase depression,[36, 41, 149] whereas the dog showed a 25% depression[74] in plasma cholinesterase when fed at 1 p.p.m. The goat may, however, be somewhat more sensitive.[174] It seems safe to predict that the influence of rumen microorganisms will be shown only when organophosphates are given in the feed or as an oral dose. With dermal or other routes, rumen effects should be negligible. The effects will always be such as to reduce the expected toxicity, and should be most marked with compounds containing a *p*-nitrophenyl substituent.

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CHAPTER 7

Effects in Insects*

Results of Poisoning

SYMPTOMS

Organophosphates usually induce the following symptoms: increased irritability, then hyperexcitability, then tremors of the whole body (most marked in the extremities), and finally paralysis and death. The course of these actions varies with dose, compound, and route of administration: usually higher doses produce more rapid effects. In the cockroach, phosphates (such as TEPP and DFP) usually cause violent symptoms within a few minutes, whereas phosphorothionates, such as parathion, may show no effects for hours, e.g., 2 hours at three times the LD_{50}, 7 hours at the LD_{50}. This difference is most marked with injection, and less with topical applications. Except with very high doses, death is long delayed: in TEPP it comes 18–26 hours after poisoning, in parathion 21–36 hours after poisoning, and in aminoparathion 24–45 hours after poisoning.[20] In the housefly these effects are compressed: hyperexcitability is usual in 30 minutes, paralysis in 3 hours, and death in 24 hours.[67] In malathion poisoning of the American cockroach, although symptoms develop in average times, death is extraordinarily delayed by about 5 days at the LD_{50}.[47, 83]

Perhaps as a consequence of hyperactivity, parathion and TEPP induce in German cockroaches an elevated respiratory rate (about 3 times normal at the peak which is at about 2 hours) which falls off as paralysis sets in.[36] This elevation is not found with malathion.[83]

INHIBITION OF CHOLINESTERASE

The first experiments on organophosphate poisoning of insects were done by Chadwick and Hill in 1947.[19] In a model study, they showed that minute quantities of DFP and a TEPP preparation (and also the carbamate eserine) inhibited insect cholinesterase *in vivo* and *in vitro*. As Fig. 7.1 shows, there was a close parallel between the cholinesterase inhibition *in vivo* by a given dose and the per cent of mortality. Other experiments showed that there was no excess inhibitor in the nerve cords at the time of homogenization, so the data give a true picture of *in vivo* conditions.†

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* The few pieces of data on mites will also be included in this chapter.

† Chadwick (private communication, 1960) has since had reservations about the implications of Fig. 7.1. He points out that one would expect rather that if cholin-

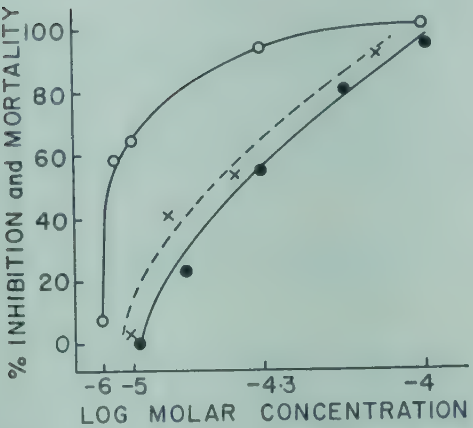

Fig. 7.1. Effects of DFP on the American cockroach. From Chadwick and Hill.[19]
—○— = Cholinesterase inhibition *in vitro*
- -×- = Cholinesterase inhibition *in vivo* after injection
—●— = Mortality after injection

Next came the famous study of Metcalf and March in 1949.[74] Their *in vivo* work with parathion and TEPP on the bee showed a correlation between symptoms and cholinesterase inhibition; in general, 50 % inhibition led to hyperexcitability, 65 % to knockdown, 90 % to prostration, and 98 % to death. More extensive observations *in vivo* were made by Chamberlain and Hoskins[20] who used 18 organophosphates on the American cockroach. Their data showed that rapidly toxic compounds (TEPP and paraoxon) caused rapid cholinesterase inhibition of the order of 90 % in 30 minutes for doses near the LD_{50} ; slowly acting compounds (diethyl phosphorochloridate and parathion) caused extensive inhibition only after some hours. They also noted that the cholinesterase of cockroaches treated with sublethal doses of TEPP recovered from 85 % inhibition at 15 minutes to 35 % at 24 hours; such studies were not made for other compounds.

That the cholinesterase of poisoned insects can recover readily was later shown for malathion[83] and for TEPP.[107] An unusually extensive study of the phenomenon was made by Mengle and Casida,[67] some of whose results are shown in Fig. 7.2. Clearly the LD_{50} dose of all compounds leads to a rather rapid and extensive inhibition of cholinesterase, yet within 21 hours the recovery of enzyme in survivors is complete. It should be noted that these results are from a shifting population of flies; at 5 minutes all flies are sampled, but by 1280 minutes only half are alive and therefore sampled.

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esterase inhibition is the sole cause of death, all those having more than a certain amount of their enzyme inhibited would die, the rest would live; yet it seems that some die with only 10% of their enzyme inhibited, some survive with 90% inhibited. Furthermore, the per cent inhibition found *in vivo* varies with the time between treatment and assay, as will be seen below. The apparently excellent correlation of Fig 7.1 may, therefore, be fortuitous.

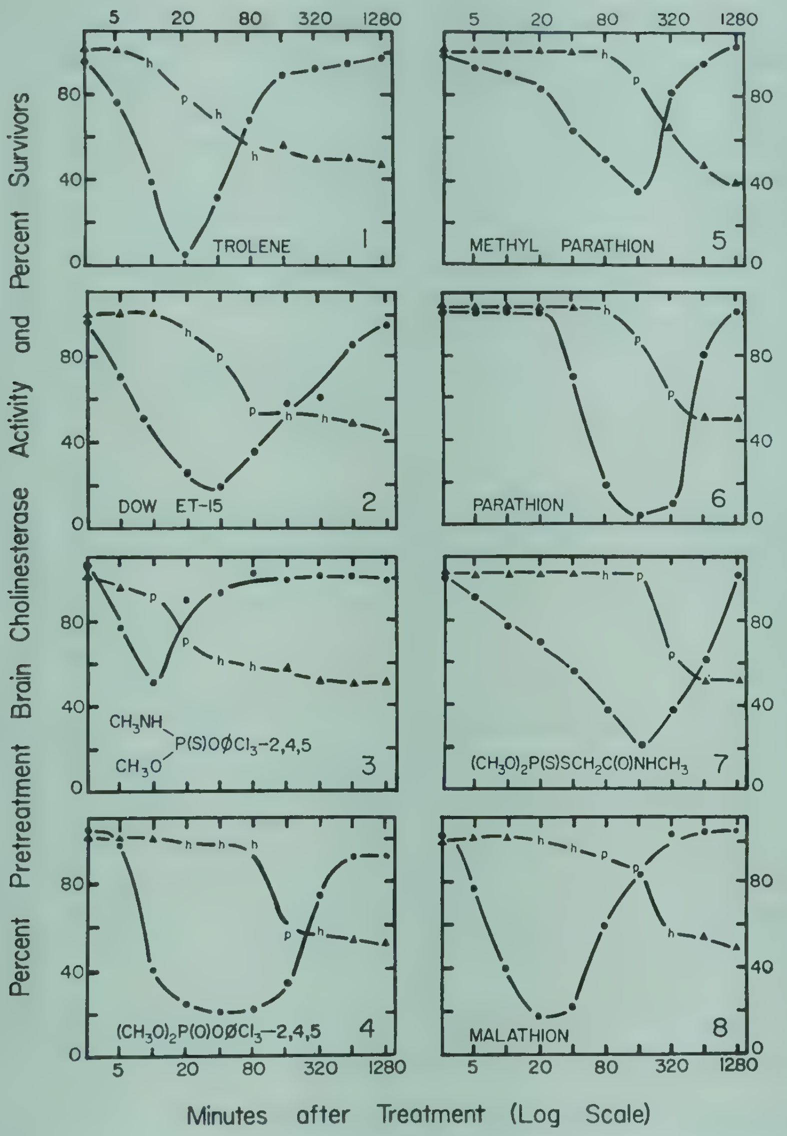

FIG. 7.2. Effects at varying times of organophosphates upon surviving houseflies. From Mengle and Casida.[67] Dose: 24 hours LD_{50}

●—● = % of pretreatment head cholinesterase activity
▲—*h*—*p*- = % survivors; *h* = hyperactive, *p* = paralyzed

Since O'Brien showed with malathion[83] that when the whole population was sampled, recovery to 50% of normal enzyme activity occurred, presumably the activity of the dead flies is reduced to zero and stays there. At the trough of activity, the enzyme of dead or doomed flies is fully inhibited and the others are still inhibited about 80% yet recover completely within half a day. Even with DFP, an almost complete 1-day recovery is seen.[68] In the American cockroach, similar recovery was found with malathion poisoning.[85]

Such results show enormously more rapid recovery than in the mammal, as a comparison with Chapter 6 reveals at once, particularly with respect to DFP. Yet the spontaneous recovery *in vitro* of insect cholinesterase is negligible after inhibition by organophosphates (page 101), whereas in the mammal a substantial spontaneous recovery is found. The difference between *in vivo* and *in vitro* recovery is not attributable to a difference in the nature of the inhibited enzyme. If housefly cholinesterase is inhibited *in vivo* and then the heads are homogenized, the behavior of the cholinesterase is precisely like that inhibited *in vitro*: it does not recover spontaneously, it can be reactivated by oximes, and it shows aging (page 108).[68] The difference must therefore be due to some factor present *in vivo* but not *in vitro*. Mengle and O'Brein[68] have provided evidence that live flies did indeed contain a factor capable of inducing recovery of inhibited cholinesterase. The factor was very labile, since it disappeared from homogenates within 30 minutes in the cold. It must be absent in flies killed by organophosphates. (A reactivating factor was shown in mammalian sera by Neubert *et al.*[80])

Several of the results above are open to the criticism of a possible "homogenization artifact": perhaps *in vivo* there is organophosphate present but out of contact with cholinesterase; upon homogenization, contact is made, and one obtains an illusively high estimate of the cholinesterase inhibition. (This problem has been considered with respect to mammals in Chapter 6.) The most significant formulation of this criticism has been by van Asperen[3, 4] who used substrate in his homogenizing medium to prevent the possible artifact. This "protective technique" showed small cholinesterase inhibitions in the head at the time of knockdown by DDVP, parathion, paraoxon, Diazinon, or Co-ral. The average was 33% inhibition. In the thorax-plus-abdomen, an average of 60% was found. Unfortunately, no direct comparison of protective and orthodox techniques was presented.

How important is the protective technique? van Asperen[3, 4] found that after treatment with DDVP vapor to knockdown, enough free DDVP was present to give severe interference with the assay. Colhoun[29] showed that after topical TEPP treatment, significant levels of free TEPP existed in American cockroach nerve cords up to (but not after) 7 hours from treatment. By using the protective technique, 70% inhibition at 0.5 hours was found instead of 100% with the standard technique.

But similar determinations showed no DFP in the cords of DFP-treated cockroaches[19] and Mengle[68] could duplicate his former results[67] when he used the protective technique with malathion, parathion, DFP, and dimethoate. Comparison of Figs. 7.2 and 7.3 for parathion also show that Mengle and Casida[67] and van Asperen[3] obtained similar results with their different techniques. Rasmuson and Holmstedt[98] found evidence for excess

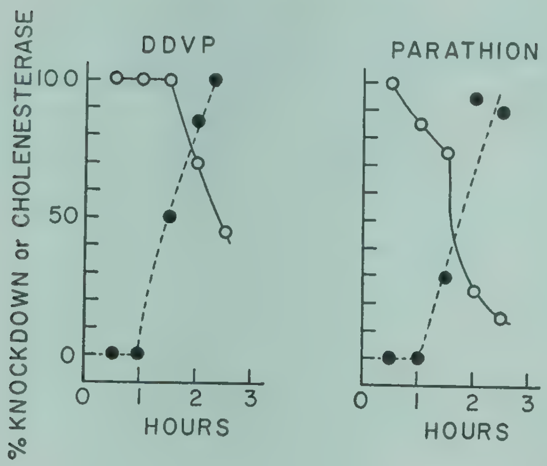

FIG. 7.3. Effect of DDVP and parathion upon knockdown and cholinesterase of whole houseflies. Flies were exposed to vapors of the insecticides. Plotted from data of van Asperen.[3]

○—○ = cholinesterase, per cent of normal
●—● = % knockdown

of inhibitor in flies poisoned by paraoxon, but not in the case of parathion and tabun. In summary, a danger may exist when phosphates are used (and is most severe soon after treatment) but does not exist with phosphorothionates.

Histochemical procedures avoid any possibility of homogenization artifacts. Winton *et al.*[124] showed that high doses (about 20 μg./gm.) of paraoxan or TEPP eliminated the cholinesterase of the American cockroach in those areas where the histochemical technique could work, i.e., wherever the nerve sheath was fractured.

ACCUMULATION OF ACETYLCHOLINE

Acetylcholine is not toxic to insects[40, 119] and is ineffective against insect synaptic transmission;[100] in the mammal it is poisonous and disrupts the synapse. There was therefore a possibility that the pharmacologically active material demonstrated in insects by many early workers[44] was not acetylcholine. However, studies with the bee,[9] the housefly,[22] and the blowfly[51] have demonstrated unequivocably by combinations of chromatography and pharmacological assay that acetylcholine is indeed present. There is also evidence for the blowfly[106] and cockroach[21] that acetylcholine accounts for all of the material assayable by the frog rectus abdominis muscle. In all the studies described below, this muscle was used as the sole assay system. The failure of acetylcholine to kill or disrupt conduction in insects is fully explained by the ion barrier protecting their nervous system (page 162).

In the mammal, small amounts of acetylcholine accumulate as a con-

TABLE 7.1. EFFECT OF ORGANOPHOSPHATES UPON LEVELS OF ACETYLCHOLINE[a]

Tissue	Organo-phosphate	Dose μg. per animal	Time after dose (hours)	Acetylcholine % increase
Fly head	TEPP	10	4	112
Fly head	Malathion	10	4	43
Fly head	Parathion	10	4	72
American cockroach nerve cord	TEPP	5	0.5	53
Mouse cerebrum	TEPP	16	0.5	22

[a] Data of Smallman and Fisher.[107]

sequence of cholinesterase poisoning (Chapter 6). In the insect, large increases may be produced by massive doses of organophosphates, e.g., to 212% of normal in the heads of houseflies treated with 10 μg./fly of TEPP (Table 7.1).

The time sequence of acetylcholine production has received much attention. The first study by Lewis and Fowler in 1956,[52] showed that in the blowfly treated with a high dose of DFP, although there was an over-all increase in acetylcholine, the level in the head actually fell. Probably this phenomenon was due to the transfer of acetylcholine from the head to the thorax and abdomen, since the rises in these parts were very marked; this conclusion was also suggested by their demonstration that if the flies were decapitated, the severed heads lost only 20% of their acetylcholine instead of about 75% in intact flies.

Smallman and Fisher,[107] in an extensive study, confirmed the effects of DFP on the housefly, but showed that they were atypical. With malathion, parathion, and TEPP no fall in the acetylcholine of the head was found; in both the trunk (= the decapitated fly) and head great increases were found, particularly with parathion (Fig. 7.4). Two other points of interest can be seen from the figure. (*a*) With TEPP and malathion, the acetylcholine level fell after 8 hours; this effect was even more pronounced at later times. The authors suggest that at death, acetylcholine in the bound form becomes free and can be hydrolyzed by the cholinesterase which (by this time) is partially recovered. (*b*) With DFP, the acetylcholine level in the whole fly is remarkably constant and fairly low; this constancy was maintained for 36 hours. The authors feel that inhibition of choline acetylase by DFP may be the cause.

What is the source of this great excess of acetylcholine observed in organophosphate poisoning? Colhoun[26, 28] has pointed out that the acetylcholine may be produced in two ways: that which is released from the bound form during synaptic transmission, escaping the hydrolysis which is

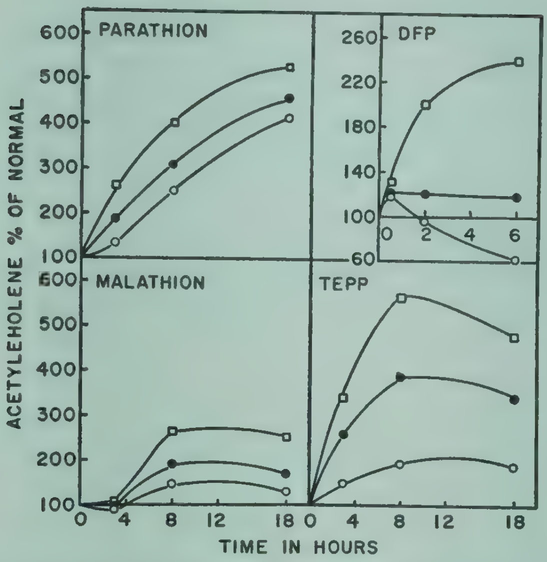

FIG. 7.4. Effects of organophosphates upon acetylcholine levels in houseflies. From Smallman and Fisher.[107] Dose about 50 μg./gm.

● = whole fly; ○ = head only; □ = trunk only.

its usual fate, and that which may be produced by fresh synthesis quite unrelated to nervous activity. In the American cockroach, Colhoun has evidence of both sources being utilized in consecutive phases (Fig. 7.5). After TEPP poisoning, a small transient rise in acetylcholine in the nerve cord is seen which subsides in 4 hours. This is attributable to nervous release which subsides as hyperexcitability (and greatly stimulated nervous activity) gives place to paralysis. Secondly, an enormous increase is developed which subsides after about 2 days as total necrosis sets in. This second peak coincides with electrical silence in the cord. As Fig. 7.5 shows, this second peak is found even with DDT, which has no effect upon cholinesterase.

In the case of the studies with blowflies and houseflies, we have no way of knowing which of these two acetylcholine sources is being tapped at any particular time. It is probably safe to assume that most of the acetylcholine produced during the first few hours is attributable to high nervous activity coupled with inhibited cholinesterase.

These studies on acetylcholine should largely remove serious doubts that the cholinesterase of poisoned insects is effectively inhibited. It is true that the great increases in late poisoning might be dependent upon prior necrosis of nerve tissue, and therefore not of importance in causing death (if we accept the fairly safe assumption that such necrosis is itself a sufficient cause of death). But the early prenecrotic rises of acetylcholine suggest that cholinesterase must be severely depressed at that time. The word "suggest" is used advisedly—we cannot exclude the possibility that

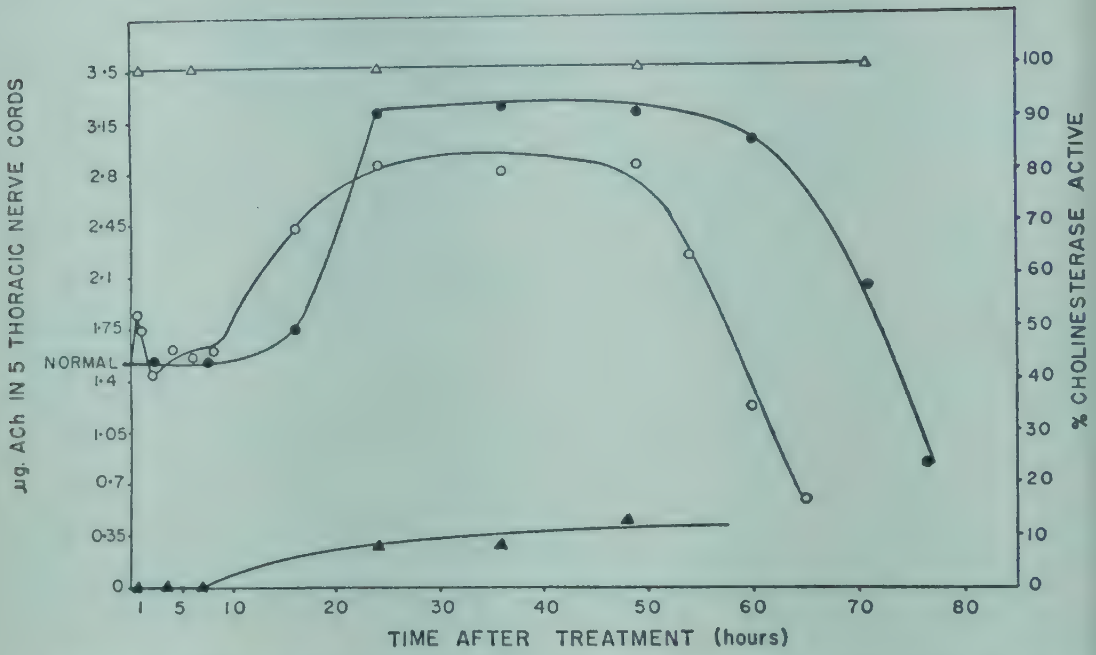

Fig. 7.5. Levels of cholinesterase and acetylcholine in the thoracic nerve cords of American cockroaches treated with TEPP (5 µg./gm.) or DDT (50 µg./gm.). From Colhoun.[28]

○ = acetylcholine after TEPP
● = acetylcholine after DDT
▲ = cholinesterase after TEPP
△ = cholinesterase after DDT

acetylcholine is synthesized in tissues outside the nervous system, and consequently is kept out of contact with cholinesterase by the "nerve barrier." This possibility is minimized by Colhoun's finding that in the American cockroach choline acetylase (the acetylcholine-synthesizing enzyme) is restricted to nervous tissue.[27]

We now have concrete evidence in favor of the chain of events in organophosphate poisoning of insects: cholinesterase inhibition, thence excess acetylcholine, thence nervous system disruption (? by depolarization caused by acetylcholine) and death. We will now consider some other factors that could be important.

Other Effects; the Cause of Death

The early studies in 1947 and 1949 on the action of organophosphates in insects led to the conclusion that the cause of death was the inhibition of cholinesterase.[19, 74] The hypothesis is still the most probable one, yet there are a number of uncomfortable anomalies which should make one reluctant to raise the hypothesis to the status of a theory. At present, no one has suggested any other system which is substantially inhibited in poisoning and has any demonstrable importance in the insect. We will now consider some of the real and apparent anomalies which may throw some doubt on the "cholinesterase hypothesis."

The first doubts were raised by Lord and Potter in 1950.[54] They pointed to the poor relationship between toxicity and *in vitro* anticholinesterase activity, contrary to Metcalf and March's view[74] that "in general there is a direct relationship between *in vitro* cholinesterase inhibition and *in vivo* toxicity." However, both these views were expressed before it was realized that phosphorothionates and phosphoramidates have to be activated *in vivo* before they are effective anticholinesterases; the *in vitro* data have, therefore, little significance except with phosphates. Lord and Potter's observation[54, 55] that cholinesterase was not apparent (with preliminary experiments) in certain insect material, e.g., whole *Tribolium castaneum*, was of course crucial for the "cholinesterase hypothesis," but after independent studies had brought it into question[82] it was later corrected.[56] It therefore remains true that cholinesterase has been found in all adult and larval insects examined.

Lord and Potter were also the first to suggest that esterases other than cholinesterase may be important in organophosphate poisoning.[54, 55] First they studied the hydrolysis of *o*-nitrophenyl acetate and found that it occurred in all their insect preparations; it was inhibited *in vitro* only by high concentrations of TEPP. In 1954 they compared the hydrolysis of acetylcholine and of phenyl acetate in four adult insects.[57] Hydrolysis of the aromatic ester *in vitro* was sensitive to low concentrations of organophosphates. In fact, taking four inhibitors (TEPP, paraoxon, and two parathion isomers) with the four insect preparations, in 11 out of 16 cases the hydrolysis of phenyl acetate was more sensitive than that of acetylcholine. This certainly indicated that this esterase activity deserved attention; however no physiological role could be assigned to phenyl acetate, whereas with acetylcholine one could (by analogy with the mammal) suppose a vital role in nervous transmission.

But is this analogy with the mammal a fair one? In 1954 Hofp[40] confirmed an earlier observation that acetylcholine is nontoxic to insects even in massive doses (about 1000 mg./kg.) whereas it is known to be toxic to mammals (e.g., intravenous LD_{50} to the mouse = 20 mg./kg.[77]). Moreover, several other compounds of related significance showed similar differences from the mammal: carbamoylcholine, prostigmine, and atropine were virtually nontoxic to insects. Hopf concluded that it was doubtful whether insecticides do act as cholinesterase inhibitors. This powerful blow at the "cholinesterase hypothesis" was resolved some years later, when it became apparent that exogenous acetylcholine simply failed to penetrate to vital sites because of an ion barrier (see pages 161–170). O'Brien and Fisher[85] showed that ionized anticholinesterases were always nontoxic to insects (5 species studied) in spite of their toxicity to mammals. Of the unionizable anticholinesterases, only schradan (and perhaps other phosphoramidates) is toxic to mammals but not to many insects, and this is due to the fact that it too fails to penetrate the insect nerve cord.[88] Many other ionizable compounds which are neurotoxic agents in mammals are ineffective against insects, probably for the same reason—failure to reach the site of action.[85] These observations also account fully for the failure of atropine to act in insects as an antidote to organophosphates:[39] atropine has a pK_a' of 9.3, and is therefore 99.5% ionized at pH 7. This matter is considered further on pages 329–334.

Hopf[40] next explored the possibility which Lord and Potter had opened that organophosphates might owe their action in insects to inhibition of some enzyme other than cholinesterase. Using four phosphates with the locust nerve cord, he found a close parallel between the inhibition (both *in vivo* and *in vitro*) of acetylcholine and *o*-nitrophenylacetate hydrolysis, suggesting that perhaps only one enzyme was involved; he showed that in horse serum by contrast, at least two enzymes were involved. The choice of serum for comparison was not a happy one, as it is well-known for containing a number of enzymes, and its cholinesterase is different from that of nerve tissue (page 9) and of little significance in organophosphate poisoning (page 188). Mammalian brain cholinesterase would have been better. In fact, Mounter and Whittaker[79] had shown in 1953 that purified true cholinesterase (from erythrocytes or cobra venom) hydrolyzes phenyl esters about as well as acetylcholine. Hopf's results seemed therefore to confirm the well established similarity[125, 126] between acetylcholinesterase of mammals and insects.

Casida[17] and Hopf[40] studied the inhibition of *o*-nitrophenyl acetate and acetylcholine hydrolysis in the nerve cords of insects poisoned by organophosphates. Table 7.2 shows the results. In seven cases out of nine, acetylcholine hydrolysis was affected more than that of *o*-nitrophenylacetate, but the differences were seldom striking; in general, the sensitivities could be said to be surprisingly similar. Casida extended his observations to six other tissues in the American cockroach; the results were quite similar to those for the nerve cord.

More recent studies by Metcalf *et al.*[76] and van Asperen[6] using various combinations of substrates and inhibitors have shown that insects (bee, housefly, and American cockroach) contain (*a*) an acetylcholinesterase whose properties are in many respects similar to that of mammals; (*b*) an aliesterase which is labile and eserine-insensitive but organophosphate-sensitive; (*c*) an aromatic esterase which is eserine-insensitive and organophosphate-insensitive, and is present mainly in the body (as opposed to the head) of the bee and cockroach. All three enzymes could hydrolyze aro-

TABLE 7.2. HYDROLYSIS OF ACETYLCHOLINE AND *o*-NITROPHENYLACETATE IN NERVE CORDS FROM POISONED INSECTS[a]

Phosphate	Dose (μg./gm.)	Insect	Time after treatment (hours)	Per cent Inhibition of hydrolysis of	
				Acetylcholine	*o*-Nitrophenyl acetate
TEPP	4	locust	4	86	76
Diethyl 4-chlorophenyl phosphate	1000	locust	4	70	30
Diethyl 2-chlorophenyl phosphate	1000	locust	4	51	31
Diethyl 2,4-dichlorophenyl phosphate	100	locust	4	75	89
Diethyl 2,4,5-trichlorophenyl phosphate	10	locust	4	64	86
Paraoxon	2	American cockroach	1	67	49
DFP	50	American cockroach	1	77	73
Dipterex	500	American cockroach	1	77	71
Phosdrin (mixed isomers)	50	American cockroach	1	85	37

[a] Data of Hopf[40] for locust; data of Casida[17] for cockroach.

matic esters! To differentiate between them, one can use eserine and phosphates. It may be, of course, that these three enzymes represent groups of enzymes. What is certain is that aromatic esters are particularly unsuitable substrates for studying enzyme inhibition if any degree of specificity is desired, unless some device is used to distinguish between the three enzymes.

Just when the cholinesterase hypothesis was appearing to be the victor, another anomaly was found. Van Asperen[4] showed, in 1958, that when flies were knocked down by DDVP, their aliesterase was inhibited by 83%, but their cholinesterase by only 27%. In this work a "protective" procedure was used (page 244), so there can be no question of artifacts. Substantially similar results were found with parathion, paraoxon, Diazinon, and Co-ral.[3] Subsequent work by O'Brien,[81] at various times after poisoning, has shown similar effects: houseflies were treated with the LD_{50} dose of parathion, malathion, Diazinon, and Co-ral, and at various times the ability of homogenates to hydrolyze acetylcholine, methyl butyrate, phenylacetate and tributyrin was studied, using, in all cases, a protective procedure.* In every case, a steady loss of enzyme activity occurred up to 1 hour, then a steady recovery occurred. The maximum inhibition was about 60% for cholinesterase (i.e., for acetylcholine hydrolysis) and from 85–95% for hydrolysis of the other substrates. Maximum inhibition occurred at the same time for all substrates, and preceded knockdown by about 1 hour.

These studies appeared to weaken the case for the cholinesterase hypothesis. However, in 1959, van Asperen and Oppenoorth[7] found that resistant flies had much less aliesterase than susceptible flies: the difference was twofold for tributyrin hydrolysis, and up to sixfold for methyl butyrate. They concluded that it was unlikely that aliesterase inhibition was an important factor in organophosphate poisoning. The same argument presumably holds for the phenylacetate hydrolyzing enzyme(s).

Another important piece of evidence against the importance of aliesterase inhibition was provided by Stegwee:[116] he found that with triorthocresyl phosphate (TOCP) treatment of houseflies, he could profoundly inhibit the aliesterase without affecting the cholinesterase: the flies showed no symptoms of poisoning. Although the TOCP, used at 90 μg. per fly, had no effect upon cholinesterase, it increased the ability of subsequently applied TEPP to inhibit cholinesterase. Thus TEPP alone, at 0.2 μg. per fly, produced 51% inhibition of cholinesterase, but if applied 1 day after TOCP, it produced 67% inhibition. A very similar phenomenon *in vitro* was found by van Asperen and Oppenoorth:[8] homogenates of flies with a natural or induced low aliesterase activity had their cholinesterase more inhibited by a given concentration of DDVP, paraoxon, or "diazoxon" than homogenates from flies with a normally high level of aliesterase. The effect was attributed to the two enzymes competing for inhibition by the low levels of phosphate used(10^{-8}–10^{-9} M). Presumably, if more phosphate were used, the competition would disappear.

Colhoun[25] observed that the American cockroach was affected by TOCP much as Stegwee had reported for the housefly. Even at 1 mg. per insect, TOCP produced only lethargy, with no deaths. Yet at doses of 75 and up to 200 μg. per insect, aliesterase was inhibited 100% and cholinesterase not at all. Nevertheless, TOCP increased the effectiveness of subsequently applied TEPP, reducing the average knockdown time from 60 to 22 minutes.

It seems safe to conclude that aliesterase inhibition is of little importance in organophosphate poisoning. Probably the reason why it is so readily inhibited in the housefly is that 96% of it is outside the nervous system and, therefore, is readily available for inhibition; by contrast, 91% of the cholinesterase is inside the nervous system.[116]

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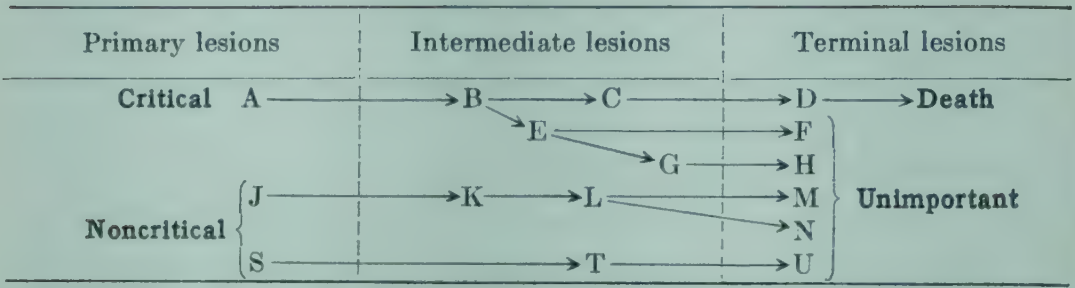
* Recent work by van Asperen and Oppenoorth[8] has shown that methyl butyrate and perhaps tributyrin are poor protecting substrates against paraoxon and "diazoxon," although they are effective for DDVP.

Winteringham's group[121, 122, 123] have studied effects of DFP upon carbohydrate and acetylcholine metabolism, using their "labeled pool technique": C^{14}-acetate is injected into houseflies, with or without DFP. At various times thereafter, the insects are extracted and the amounts of the various C^{14}-labeled metabolites are found. Glutamine labeling was thus increased, e.g., threefold at 5 hours, whereas glutamate plus proline was somewhat reduced, e.g., 10% at 5 hours. Similar experiments with a P^{32}-labeled pool showed no effects of DFP upon phosphorylated compounds. These results are somewhat disappointing; such changes as were found are probably concomitants of the transiently stimulated respiration which these workers show for DFP.

More interesting was their observation[123] that DFP actually reduced the rate of acetylcholine synthesis from C^{14}-acetate *in vivo*. This can have little importance in poisoning, since ample free acetylcholine is produced in DFP poisoning (page 247).

Let us now draw together some of the observations on insects given throughout this book, and try to find precisely what we do know about the cause of death in their poisoning by organophosphates. But first we must decide (*a*) what is death, and (*b*) what kind of data can establish its cause. With insects, the criterion of death often used is the absence of spontaneous or inducible movement; since such a state could in fact represent paralysis of the extremities, it is not a good criterion. Another criterion would be cessation of respiration, as judged by oxygen uptake; but it is likely that tissues will continue to respire even when the organism, considered as a whole, is dead.

If the primary lesion is inhibition of a particular enzyme, this leads mediately or immediately to the terminal lesion (which would presumably also be biochemical if respiratory cessation were the criterion). Between the primary and terminal lesion there may well be numerous other steps in the causal chain; for example, in mammals the primary lesion, cholinesterase inhibition, leads to two physiological lesions (nerve block, then failure of the respiratory apparatus), then to anoxia, and hence death. Almost certainly the poisoning will have other primary lesions, which have either no consequences or else unimportant consequences; thus, in the mammal undoubtedly some chymotrypsin inhibition will occur with acute DFP poisoning; the consequent mild interference with digestion can have no importance. We may expect also that the critical primary lesion besides initiating the chain of events leading to death, will initiate other chains leading to unimportant consequences. This is shown here diagrammatically. We cannot exclude the possibility that two or more of these chains of



events may interact to cause death; for instance the nonlethal lesions F or U in the diagram might increase the effectiveness of the critical terminal lesion D. Also, any of the lesions may give rise to a symptom (e.g., paralysis, hyperexcitability). Only those caused by lesions in the significant chain (A, B, C, or D) are of importance. We must be prepared to find a complex series of chains in the case of organophosphate poisoning, since we are dealing with agents of fairly high chemical reactivity; with a less reactive and more specific antimetabolite, e.g., fluorocitrate, we would expect a similar picture.

When we attempt to establish the mode of action of a poison, our major interest is to trace the significant chain A–B–C–D in the diagram. But in the poisoned animal we will also detect the other lesions shown. How do we distinguish between significant and insignificant events? There is no certain way. Some of the tests might be: is there a correlation between the time of the lesion and the symptoms? (However, there may be a time lapse before the symptom develops.) Does the lesion occur in a sequence of biochemical or physiological events of known importance? (This may presuppose a greater knowledge of such events than we have.) Is the lesion caused by the toxic agent, not by nontoxic congeners? Is there a correlation between toxicity and extent of lesion for a series of compounds? Is there a threshold value for the extent of a lesion which, once exceeded, produces death? Is susceptibility to lesion-production in various species or strains correlated with susceptibility to lethal poisoning by a given agent?

Let us now return to the specific problem, and apply some of the above tests to the "cholinesterase hypothesis."

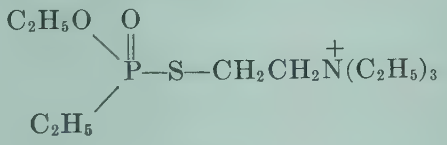
(*1*) *Time Correlations.* The extensive observations of Mengle and Casida[67] with 17 organophosphates (8 of which are shown in Fig. 7.2) against the housefly showed quite good coincidence, in about 12 cases, between the point of maximal inhibition of cholinesterase and the time of onset of deaths. In the case of malathion the correlation was extremely poor (Fig. 7.2) as O'Brien had pointed out earlier; he observed in houseflies that when deaths began, the average cholinesterase had recovered to 45% of normal (from its low of 2% of normal). In the American cockroach, a similar effect was seen. Malathion seems to be an unusual organophosphate in other respects, e.g., its failure to affect oxygen consumption and its extreme slowness in poisoning, and it may be that factors other than cholinesterase inhibition play a part—although a search for such factors was not successful.[83, 84]

It is important that although the time correlations are poor in some of Mengle and Casida's results, extensive deaths never *preceded* substantial cholinesterase inhibition; if such a case is ever shown, it will be strong evidence against the "cholinesterase hypothesis." But in data such as Fig. 7.2, we always have the possibility that the inhibition of some other enzyme or enzymes is paralleling the course of cholinesterase, particularly in phosphorothionates, where the peak of inhibition may only be a reflection of the peak of oxygen analog formation.

(*2*) *Correlation of Toxicity and Antienzyme Activity.* Metcalf and March[74] first investigated this correlation in 1949. However, this excellent study is outdated by our current knowledge that scrupulous care is needed in purifying these compounds, and that phosphorothionates and phosphoramidates are not active per se. If, with this last point in mind, we examine only the phosphates reported, the correlation between insect toxicity and *in vitro* anticholinesterase activity leaves a good deal to be desired, although it is qualitatively evident. But quite apart from purity considerations, any compound which can be detoxified *in vivo* (and it is almost certain that all organophosphates can be to some extent) may have a low toxicity in spite of high activity against any enzyme *in vitro*.

If someone could be induced to do the work, a useful correlation would be of LD_{50} with *in vivo* anticholinesterase activity (i.e., the amount of compound needed to produce a given inhibition *in vivo*). This would also circumvent the need for scrupulous purity.

An anomaly is the finding of Hopf and Taylor[41] that the cholinesterase of locust nerve can be totally inhibited by compound (I), even though the



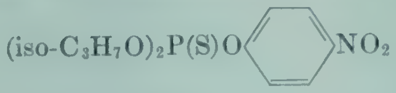
$$(C_2H_5O)(C_2H_5)P(=O)-S-CH_2CH_2\overset{+}{N}(C_2H_5)_3$$

(I)

insect is unaffected. These authors mention that the inhibition of an unspecified phenylesterase more closely paralleled symptomology (no data given). Finally, van Asperen,[3] as mentioned above, found only 27–51 % inhibition of whole fly cholinesterase at knockdown by five organophosphates, whereas with DDVP, an aliesterase was inhibited 83 %. Possibly, knockdown is a phenomenon influenced by lesions other than those which influence death.

There are totally inadequate data to decide whether these sorts of correlations fit better with cholinesterase or with some other esterase.

(*3*) *Susceptibility of Insect and Enzyme in Various Species.* Instead of using a series of compounds, one can use a series of insects for these correlating experiments. The most-quoted example is the finding of Metcalf and March that diisopropyl *p*-nitrophenyl phosphorothionate (II) is toxic to flies and inhibits fly brain cholinesterase *in vitro*, but is nontoxic to bees and does not inhibit bee brain cholinesterase.[74] This implies, at first sight, a difference in the types of cholinesterase; but it could be due to a degrading enzyme in the bee preparation, or the acidity of the bee preparation.[108][*] However, there is evidence that the cholinesterases of insects vary as widely in their susceptibility to inhibitors as do those of mammals.[18, 20, 87] Probably further investigation of this correlation with crude homogenates would be rather unrewarding, as species differences with regard to degrading and activating enzymes are likely to outweigh differences in cholinesterase or other target enzymes.



(II)

(*4*) *Threshold of Inhibition.* There may be a certain threshold of target-enzyme inhibition such that, once exceeded, death ensues. Two problems in assessing this value are the difficulty of precise assessment of *in vivo* inhibition (page 244)—this is minimal in phosphorothionates—and the possibility of local inhibition of a vital enzyme being overlooked when gross studies are made. Thus, van Asperen[3] finds that body cholinesterase may be three times as inhibited as head cholinesterase in poisoned flies. Which is the vital fraction? He feels that the body enzyme may be, since decapitated flies show a typical development of symptoms after poisoning.[5]

Chamberlain and Hoskins[20] reported that in the American cockroach poisoned by various organophosphates, symptoms only appeared if the nerve cholinesterase was inhibited 85%. A more extensive study along this line might be rewarding, examining other enzymes simultaneously.

One pitfall in studying the threshold effect is the possibility of delay between the time of reaching the threshold and the time of onset of the resultant symptom. It is quite possible, for instance, that once the cholinesterase at some particular site is 95% inhibited, death always results. But death may not occur until many hours after, and during this delay period the cholinesterase might become either more or less inhibited. In such circumstances it would be difficult to assess the value of the threshold, for the delay period would be unknown.

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[*] Besides, (II) is a phosphorothionate and therefore almost certainly is not an anticholinesterase *in vitro*; the reported inhibition must have been caused by an impurity, perhaps an isomer of (II).

In conclusion, there appears to be qualitatively good evidence in favor of the "cholinesterase hypothesis." The principal objections to it are: (*a*) Hopf and Taylor's observation that locusts survive drastic cholinesterase inhibition; (*b*) the fact that cholinesterase need not be substantially inhibited at death; and (*c*) the demonstration (page 171) of vigorous activity in isolated nerve cord soaked in TEPP. These anomalies might seem enough to neutralize the favorable evidence given above. In the writer's view, the best evidence in fact comes from the analogy with the mammalian situations (for which the hypothesis finds much better support) and from the over-all coherence of the hypothesis. There is good evidence for cholinergic functioning in insect synapses, we know that organophosphates are potent and quite specific anticholinesterases, and the symptoms of poisoning are those of nervous dysfunction. Until evidence is produced of interference with some other system of demonstrable importance in nerve function, the "cholinesterase hypothesis" will continue to provide the best available explanation of the insecticidal action of organophosphates.

It must be pointed out that even if the "cholinesterase hypothesis" is convincingly proved, we still have absolutely no information at all as to the *ultimate* cause of death in insects (in mammals asphyxiation is the ultimate cause). Assuming that the nervous system is disrupted, what follows next? Is their crude respiratory apparatus interfered with? If so, does asphyxiation or dehydration occur? On this last point, Roan *et al.*[99] observed in 1950 that after TEPP treatment, the American cockroach at prostration had lost all its free body fluids. However, Krueger and O'Brien[47] found that the relative humidity of the environment did not affect the LD_{50} of malathion to the American cockroach.

Ovicidal Action

The question of how organophosphates kill insect eggs is even more fraught with uncertainties than is the case for adult forms. In the course of egg development one sees changes in susceptibility to poisoning, content and kind of esters and esterases, and physiological function. As Smith and Wagenknecht have pointed out,[112] such a series of changes should constitute an ideal system for determining mode of action, and ought to throw light on the way in which these poisons affect the adult forms.

The early work was upon TEPP preparations; these were shown to be ineffective when concentrations of the range practicable in the field were used.[92, 129] In 1951, Lord and Potter[55] showed that TEPP could kill the eggs of the tomato moth *Diataraxia oleracea* and the Mediterranean flour moth *Ephestia kühniella.* Two conditions were established: at the LD_{50} level (0.1% spray for *D. oleracea* and 0.5% for *E. kühniella*) the embryos almost completed their development before they died; at high levels (e.g., 0.4 µl. of 15% TEPP per egg) death occurred promptly—but only if the eggs were less than 24 hours old. Parathion was far more effective, 125 times better than TEPP (by contact) against *E. kühniella* and 29 times better against *D. oleracea*. The authors pointed to the presence in these eggs of an enzyme hydrolyzing *o*-nitrophenylacetate and raised the possibility that its inhibition might be important in poisoning. However, the enzyme was fairly insensitive to TEPP *in vitro*.

Salkeld and Potter[102] studied the question of the variation of susceptibility with the age of the egg, using a TEPP preparation sprayed on the above insects and the cotton stainer, *Dysdercus fasciatus*. In every case there was a marked steady increase of susceptibility with age, particularly evident in *D. fasciatus* which was ten times more sensitive at 8 days than at 1 day (no mention was made of an increased sensitivity in the first 24 hours). But Smith[110] found that with parathion on the peachtree borer *Sanninoidea exitiosa* the age variation depended upon the mode of treatment: by direct contact the older eggs were more susceptible, by vapor treatment they were less susceptible.

Many studies upon parathion have shown the phenomenon of delayed death. The following eggs develop fully but fail to hatch: silkworm *Bombyx mori*, aphid (unspecified), apple sucker *Psylla mali*,[38] housefly,[64] and the peachtree borer *Sanninoidea exitiosa*.[110] The following emerged and died soon after: stick insect *Carausius morosus*, common winter moth *Operophthera brumata*, red spider mite *Metatetranychus ulmi*,[38] milkweed bug *Oncopeltus fasciatus*, and the Mexican bean beetle *Epilachna varivestris*.[86]* Holz[38] suggested in 1949 that this second group died by consuming parathion that was in the chorion, as they chewed their way out. This view was echoed by Schwartz[104] who found that treated Colorado beetle eggs all died with 0.1% parathion, but that with lower concentrations (e.g., 0.04%) almost all hatched and then died. Schuhmann[103] was also emphatically of this opinion in the case of the red plum maggot *Laspeyresia funebrana*: "obviously" the parathion was dissolved in the chorion and poisoned the emerging larva. Speyer[113] reached the same conclusion. However, since some of the susceptible species never emerge, and some such as *Psylla mali* have sucking instead of chewing mouth parts, the hypothesis is improbable.

In 1953 Staudenmayer,[114] studying parathion treatment against eggs of the silkworm *Bombyx mori*, found that regardless of the time of treatment, the eggs were unaffected until 2 days before hatching (Fig. 7.6). At this time the oxygen uptake was abruptly inhibited, and this inhibition grew progressively more severe until the embryos died, without hatching, about

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* It is quite likely that these two patterns represent differences in the doses used rather than real species differences.[109]

8 days after the controls had hatched. Staudenmayer treated the eggs by immersing them in emulsified 0.1 % parathion for 15 minutes.

Smith and Avens[111] had noted in 1954 that parathion was an effective ovicide for the peachtree borer, and that under practical conditions (i.e., low levels of insecticide) embryonic development was complete, but no hatching occurred. Later, Smith[110] showed precisely the same respiratory pattern for this insect as described above for *B. mori*. The time of the beginning of respiratory inhibition was only advanced 1 day by increasing the parathion dose eightfold. Smith pointed out that the delayed action of parathion could be due to (*a*) an immediate primary lesion but a delayed terminal lesion (c.f. page 256) or (*b*) a delayed primary lesion produced by parathion retention in the egg and its action upon its target enzyme later. He favored the latter view.

The work of Smith and of Staudenmayer suggests that the delayed death is not attributable to emergent larvae chewing a poisonous chorion. However, these two workers dealt only with eggs that did not hatch. It is possible that in those cases (given above) in which the larva does chew its way out, it may consume parathion in the process.

In 1955, Staudenmayer[115] found that true cholinesterase in *B. mori* eggs was absent till about 6 days before hatching, but then rose extraordinarily rapidly and uniformly; the rate fell off somewhat just before hatching. Lord and Potter[58] found that substantially the same was true of *D. oleracea*; but the phenyl acetate hydrolyzing enzyme was demonstrable in similar levels at all stages. Smith and Wagenknecht[112] found that cholinesterase in the eggs of the peachtree borer (Fig. 7.7) and the milkweed bug also showed this striking upsurge of cholinesterase in the middle of their

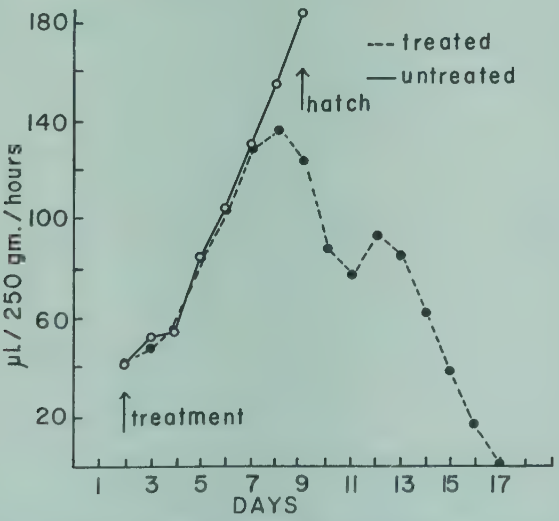


Fig. 7.6. Oxygen uptake by silkworm eggs and the effect of parathion treatment. From Staudenmayer.[114]

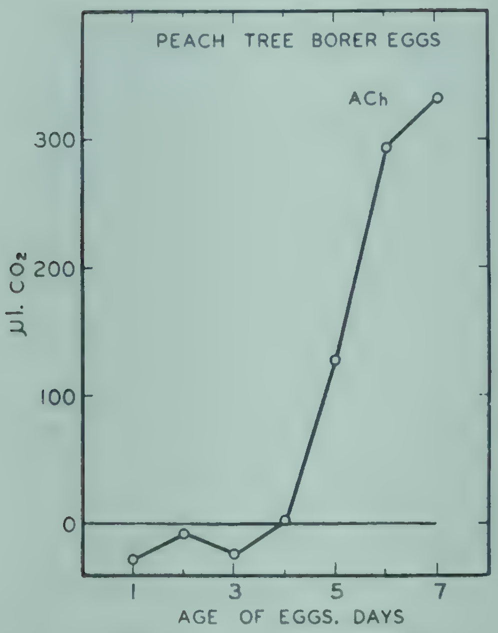


FIG. 7.7 Occurrence of cholinesterase in peachtree borer eggs of various ages. Ordinate shows μl. CO_2 released in 180 minutes by a homogenate of 2000 eggs, using manometric assay. From Smith.[110]

embryonic development: similar findings have been reported for the grasshopper *Melanoplus differentialis*,[118] the housefly,[64] the rice stem borer *Chilo simplex*, and the cabbage armyworm *Baratha brassicae*,[24] and have confirmed the data for *B. mori*.[23]

Lord and Potter[58] concluded that rapid death due to treatment with TEPP (high levels) could not be due to cholinesterase inhibition, but could be due to the inhibition of "general esterase" (a term they apply to the phenyl acetate hydrolyzing enzyme). Staudenmayer[115] felt that delayed death caused by parathion treatment (low levels) could not be due to cholinesterase inhibition, since death occurs at 2 days before hatching, whereas cholinesterase occurs 6 days before hatching.

However, Smith and Wagenknecht[112] produced evidence that peachtree borer eggs treated with parathion by fumigation on their first day had their fifth day cholinesterase inhibited by 57%, thus showing that parathion does indeed persist in these eggs and affects subsequent cholinesterase. But since the cholinesterase is inhibited so early and death occurs days later, does this constitute evidence of the unimportance of cholinesterase? No, say Mehrotra and Smallman.[65] In 1957, these authors studied cholinesterase and acetylcholine levels in the housefly egg, with and without parathion treatment; these eggs normally hatch 11–12 hours after oviposition. Their results (Table 7.3) show that acetylcholine is produced after cholinesterase in untreated eggs, and that the acetylcholine level is increased by parathion. Now the customary picture of the consequences of cholinesterase inhibition is that acetylcholine accumulates and actually causes the nervous disruption. Clearly, this cannot happen until there is plentiful production of acetylcholine, and the results of these authors suggest that this condition is not achieved until about the tenth hour; they proposed that this delayed rise in acetylcholine explained the delayed toxic action of parathion.

It must be commented that the level of acetylcholine in untreated eggs (Table 7.3) is extremely high, and the per cent increase caused by parathion (51%) is rather small compared with that found in adults (Fig. 7.4). One would like to see more extensive observations and some statistical treatment.

Substantially similar findings were reported in 1959 by David[31] for the large white butterfly *Pieris brassicae.* These eggs normally hatch in about 7 days. In untreated eggs, cholinesterase appeared at 2–3 days, and acetylcholine at 5 days. Parathion treatment killed the eggs eventually but (as in the peachtree borer) development continued up until hatching. Parathion treatment (via the cabbage plant) increased the acetylcholine level, e.g., at 6 days it was increased from a normal level of 10 to a treated level of 23 μg./gm. In one experiment the acetylcholine level of the control eggs remained at 10 μg./gm. at 7 days, whereas that in the treated eggs rose to 76 μg./gm. This suggests that extremely high levels of acetylcholine coincided with death (or rather with cessation of development).

The delayed toxic action of organophosphates to housefly and *Pieris* eggs is thus satisfactorily explained. Is a similar explanation satisfactory for other insects? We have seen that it is generally true that cholinesterase appears halfway through embryonic development, and that with moderate doses of insecticides the embryos are killed just before hatching (by "just before hatching" is implied 1 or 2 days before hatching in, say, an egg with

TABLE 7.3. EFFECT OF PARATHION UPON CHOLINESTERASE AND ACETYLCHOLINE IN HOUSEFLY EGGS[a]

Age of eggs (hours)	Acetylcholine content		Cholinesterase activity	
	Untreated	Treated	Untreated	Treated
1	0	0	0	0
3	0	0	0	0
5	0	0	0	0
7	0	0	9	7
9	5	7	9	5
11	490	742	16	5

[a] *Treatment:* Exposure on parathion-treated filter paper. Acetylcholine content in μg./gm.; cholinesterase activity in μl. CO_2 per 30 minutes per 125 mg. (protective technique). Data of Mehrotra.[64]

a 10-day development period as in *B. mori*, or 1 to 2 hours before hatching with a 12-hour development period as in the housefly). To complete the general acceptability of Mehrotra and Smallman's proposal we would have to find that either the level or the rate of production of acetylcholine is, in general, increased just before hatching, but not very much earlier.

In fact, it does seem to be generally true that acetylcholine rises rapidly until just before hatching. This is true in spite of the fact that there are three patterns of appearance found:[128] acetylcholine is found after cholinesterase in the housefly,[65] simultaneously with it in the milkweed bug[64] and silkworm *B. mori*,[23] and before it in the rice stem borer and the cabbage armyworm.[23, 127] Furthermore, there are two orders of magnitude of acetylcholine level found just before hatching: large amounts are found in the housefly, e.g., 490 μg./gm.,[65] in the rice stem borer and the cabbage armyworm, e.g. 1,200 μg./gm.,[23] and in the milkweed bug, e.g., 200 μg./gm.;[64] very small amounts are found in *B. mori*, e.g., 6 μg./gm.[23] Yet in spite of these great variations, in every case the level of acetylcholine increases sharply up to within a day or so of hatching, so that as far as timing goes, one can account for the delayed importance of inhibited cholinesterase. However, if one simply considers acetylcholine to be the actual toxicant, the amounts needed to kill the various species must vary a great deal, for on the last day very different levels are achieved.

There is an alternative to the hypothesis that the delayed toxic effect is caused by a delayed excess of acetylcholine. Instead, one might argue that the toxic action of acetylcholine is considered to be due to its interference with nerve transmission. Therefore, besides high acetylcholine levels, the embryo must be dependent upon its nervous system before it can be killed by moderate levels of organophosphates. Presumably, the organism cannot be dependent upon its nervous system until that system is fully differentiated and functioning. It is at this moment, the first moment of dependence, that the fatal effect of cholinesterase inhibition is revealed. For an analogy, cutting through a tightrope walker's rope while he is carrying it under his arm has no effect; but when he tries to walk on his rope later on, 50 feet up in the air, death ensues.

We need, therefore, to establish the moment of dependence on the nervous system. Certainly this moment will be well after the first appearance of its constitutent parts, acetylcholine, cholinesterase, and choline acetylase.* Probably the time when the production of these constituents levels out represents the time of the complete assembly of the parts; this time appears to be shortly before hatching (e.g., ref. 23). Finally, the business of a nervous system is to enable the animal to live its free-ranging life; it is useless while the animal is in the egg. It therefore seems reasonable that essential nerve function will begin shortly before hatching.

* This has been shown for the pupating silkworm.[120]

In conclusion of this section, it seems certain that organophosphates can kill insect eggs in two ways. High concentrations kill the egg at once, regardless of the absence of cholinesterase or a functioning nervous system. This effect is probably attributable to interference with glycolytic or other respiratory mechanisms, as is the case for high concentrations in mammalian tissues (page 150). Low concentrations kill at a time related to development. Clearly they are effective only against some component of the almost fully matured embryo. There are strong indications that this component is the functioning nervous system.

The general hypothesis that organophosphates kill all animals by nerve disruption caused by cholinesterase inhibition would have had a serious setback had insect eggs proven to be susceptible except at an advanced stage.

Differential Toxicity

There is a striking difference in susceptibility to organophosphates of eggs of certain species. Smith and Wagenknecht[112] have studied this problem for the parathion-susceptible peachtree borer and the nonsusceptible milkweed bug, and compared them in several ways. Their esterase content was qualitatively similar, but about three times higher in the peachtree borer on a weight basis. In both, cholinesterase appeared at 4 days from oviposition and, in both, was equally susceptible *in vitro* to parathion (it would be desirable to know if it is also equally susceptible in both to paraoxon, the active metabolite).

However, after parathion treatment, there was a marked difference in the cholinesterase level, e.g., one concentration of parathion gave about 85% inhibition of the enzyme in the peachtree borer and about 15% in the milkweed bug. The fact that cholinesterase inhibition *in vivo* is correlated so precisely with toxicity in these two species is supporting evidence of a causal relationship between the two factors. However, it is not in itself a proof. The cholinesterase might merely be an index of the paraoxon level in the egg, and the paraoxon could attack other enzymes as well.

The uptake and metabolism of radioactive parathion were studied by O'Brien and Smith[86] in an attempt to account for the above selectivity. They exposed eggs for 2 days to a vapor concentration of parathion which would kill all the peachtree borer eggs and no milkweed bug eggs. They found that the peachtree borer egg took up about three times as much parathion per unit weight as the milkweed bug eggs. In both cases, about 90% of the insecticide was on the egg surface. The peachtree borer thus had more parathion inside it; and it had about ten times as much paraoxon inside it. The paraoxon level in the peachtree borer egg was about 23 μg./gm. at 12 hours, it rose steadily to about 47 μg./gm., and then (after removal from fumigation at 2 days) fell to 34 μg./gm. at 4 days. By contrast, the paraoxon level in the milkweed bug egg never exceeded 5 μg./gm. These results account satisfactorily for the selective toxicity of parathion to these two eggs. The matter is discussed further on p. 319.

Distribution

A number of studies have been made of the total radioactivity in various insect tissues after administration of labeled organophoaphates. In some studies the subsequent counts are made on whole undigested tissues: such results are highly qualitative. A simple solvent extraction of a tissue preparation which separates chloroform extractable materials, usually the parent compound and any toxic oxidation product, from nonextractable materials, usually hydrolytic degradation products) adds enormously to the value of such a study, but is seldom done.

Very comprehensive studies were carried out in 1950 and 1951 by the Illinois group (Roan *et al.*[99] and Fernando *et al.*[33]). Compounds such as TEPP, paraoxon, and parathion were applied topically in solvent on the neck of the American cockroach. Penetration to other parts of the body was extremely rapid, the rate depending upon the solvent: thus with TEPP, 69% entered the thorax-plus-abdomen in 5 minutes if propylene glycol was used, and 49% if benzene was used. These figures were quite similar for the other organophosphates. Within 5 minutes, about half of the dose was usually in the thorax, and the remainder about equally in the head and abdomen. By 1 hour, the level in the head had fallen to about 10%, in the thorax to about 30%, and the remainder was in the abdomen. The short term distribution of TEPP into various organs is shown in Fig. 7.8. Accumulation in the foregut was particularly extensive, and this was true of all other organophosphates tested. Such data were amplified by radioautography, which added the rather unexpected result that the Malpighian tubes were innocent of radioactivity: one might have expected them to play an active part in excretion. Even after 24 hours, the foregut (but not the blood) contained anticholinesterase material. Although it is difficult to say in view of the uncertainty of quantitation, this may imply that the foregut absorbs the compounds and probably passes them into the lumen, but does not metabolize them.

For paraoxon, it was shown that only small amounts had entered the nervous system at knockdown, e.g., 0.03 μg. of an applied 5 μg. Of this, only 8×10^{-4} μg. was bound to protein, which should include cholinesterase. This important observation shows how little of the applied dose actually reaches the target (about 0.01%), and suggests that considerable improvements in this factor might be obtainable in other compounds. It also shows how incredibly potent the compound is, once arrived at the target. Curiously enough, diethyl phosphoric acid entered the nervous system

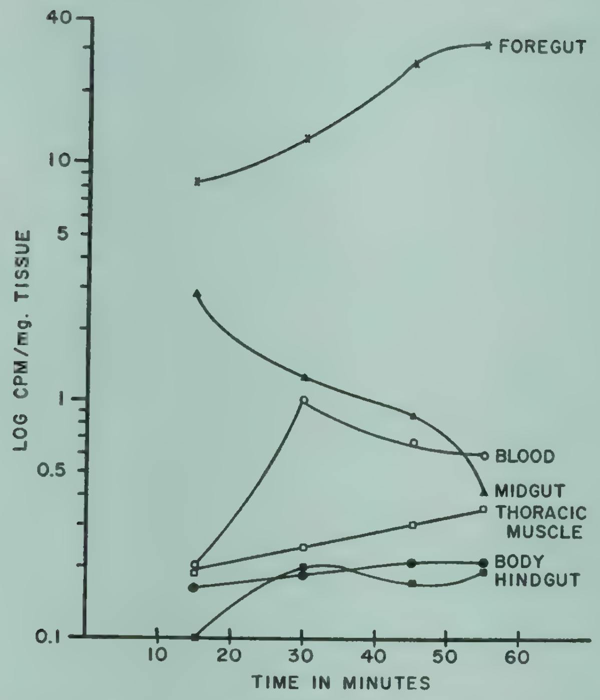
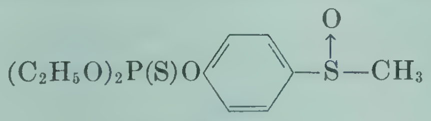


FIG. 7.8. Concentration of radioactivity in American cockroach tissues after applying labeled TEPP topically. Dose probably about 15 μg./gm. From Roan *et al.*[99]

(as well as other tissues) almost as readily as TEPP: one would have expected a profound difference caused by poor partitioning of the acid into lipids from neutral aqueous media.

Excretion of these compounds was extensive but slow: in 5 hours about 10% was excreted; in 46 hours more variation was seen, and 90% of TEPP was excreted, but only 44% of paraoxon. In the case of TEPP, it was shown that the excreta contained no anticholinesterase material nor was any radioactivity extractable by organic solvents. Presumably, all the excreted material was as hydrolytic degradation products.

Benjamini *et al.*[12] showed that with Bayer 25141 even smaller amounts of material were found in the nerve cord of the treated American cockroach:

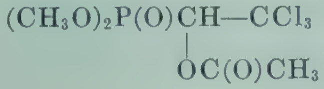


Bayer 25141

of 3 μg. applied, about 0.01 μg. were found per cord at 1 hour (assuming the cord weight was about 10 mg.). This amount fell steadily to about 0.001 μg. in 21 hours. They suggest that their 21 hour level of 0.073 μg./gm. of nerve tissue represents about 2.4 × 10^{-4} M (a huge excess for cholinesterase inhibition). This figure should be 2.4 × 10^{-7} M. Their data show an average concentration in the nerve cord (not necessarily all as the parent compound) of 2.8×10^{-6} M at 1 hour following application of 3 μg. per roach (approximately an LD_{50}). Now, when they applied the $LD_{90\text{-}100}$, i.e., 10 μg., they found 88% inhibition of cholinesterase at prostration. Assuming that at 10μg. about 3.3 times as much gets to the cord as at 3 μg., we may guess that about 9×10^{-6} M compound was in the cord at 1 hour. From their bimolecular rate constant (2.5×10^3 1 moles^{-1} minutes^{-1}), one may calculate that incubation with 5×10^{-6} M compound for 1 hour should give 50% cholinesterase inhibition if *in vivo* and *in vitro* behavior were identical.* We see then that these rough calculations give a fair estimate of the *in vivo* behavior. We do not have to assume any peculiarly uneven distribution of the compound within the nerve tissue.

Iyatomi *et al.*[42] studied DFP^{32} distribution in the American cockroach. Again, the gut was a major recipient and the nervous system a very minor recipient of radioactivity. However, in this case the Malpighian tubules contained substantial quantities of radioactivity. A partial fractionation of metabolites was attempted, but no identification was reported.

The distribution of DDVP, Dipterex, and acetyl Dipterex in the American cockroach after topical application was described by Arthur and Casida.[1] Seventeen tissues were assayed at 10, 45, and 240 minutes. Activity in the midgut was high only at 45 minutes; at 240 minutes the hindgut had taken over this activity: this is evidence that the foregut functions by passing the material into the lumen. A considerable fraction of activity was always in the blood. Curiously enough, the wings contained (per unit weight) the most radioactivity at 240 minutes! The Malphigian tubules contained substantial racioactivity at 45 and 240 minutes in the case of Dipterex and DDVP, but practically none, at any time, in the case of acetyl Dipterex. This suggests that the difference mentioned above, that DFP or its products do not enter the tubules although TEPP does, is a genuine one. This sharp division of compounds may reflect an important difference in excretion or degradation routes, and deserves serious study.



$$(CH_3O)_2P(O)\underset{\displaystyle OC(O)CH_3}{\underset{|}{CH}}\text{—}CCl_3$$

Acetyl Dipterex

The distribution of Systox isomers following administration to the American cockroach was studied by March *et al.*[63] These authors separated the products into chloroform-extractable ones and unextractable ones. In this case, the former could contain a number of products (all toxic or potentially so) since oxidations of P═S to P═O, and thioether (C—S—C) to sulfoxide (I) and sulfone (II) could all occur (page 297). Once more

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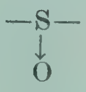
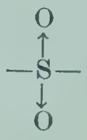
* However, the bimolecular rate constants are open to some doubt, for they disagree with the I_{50} values given in the same paper.



$\underset{\underset{\displaystyle O}{\downarrow}}{-S-}$

(I)

Sulfoxide



$\overset{\overset{\displaystyle O}{\uparrow}}{\underset{\underset{\displaystyle O}{\downarrow}}{-S-}}$

(II)

Sulfone

the foregut contained most of the activity; the majority of this was chloroform-extractable, e.g., 88% at 5 hours, after a topical dose, indicating that excretion rather than degradation is the primary function of this tissue. In the midgut, by contrast, the material was unextractable suggesting that this tissue may degrade the compounds—or possibly, that by the time the materials reached the midgut, they had been degraded nonenzymically. The blood contained little activity, never more than 2% of the applied dose, even after topical application.

The distribution of dimefox and schradan in fifteen tissues of the American cockroach was examined by Arthur and Casida.[2] One hour after treatment (at 50 μg./gm.), the malpighian tubules contained most of the dimefox; but with schradan, the hindgut contained most of the compound. The foregut was not extremely active for either compound. Degradation was extreme for dimefox (usually about 70%) but meager for schradan (usually about 14%.)

An unusual study upon parathion translocation made by Ball and Beck[10] showed that after topical application the principal route for the compound was through the nervous system. Discontinuity was created by burning, cutting, or removing parts of the nervous system either in the nerve cord, crural nerve, or cercal nerve; then parathion was applied on the side of the discontinuity distal to the brain. In every case, the time to knockdown was reduced by such operations. Clearly the nerves must have been transmitting the parathion. However, parabiotic experiments showed that the blood could distribute parathion: two cockroaches were arranged with their blood systems in contact, and topical treatment of the one then gave rise to equal amounts of parathion in the foregut of both.

Which plays the major role, parathion conduction in blood or nerve? Experiments upon the importance of the locus of application in knockdown showed that ventral applications (near the nerve cord) were far more effective than dorsal applications (near the heart), suggesting that circulation in the blood is not a particularly important factor. Supporting evidence was that blood levels of parathion were very low in poisoned cockroaches.

Metabolism

In Chapter 4, the enzymic oxidations and hydrolyses which can be accomplished by insect preparation *in vitro* were described. We shall now consider what conversions can be made *in vivo*. Unfortunately, the number of studies on this topic is lamentably small as a result of the tiny quantities of material that can be worked on. To carry out a proper time study with insects, one usually has to use not much more than an LD_{50} dose, or the animal will die too soon. Thus with dimethoate, we use 0.5 μg./gm. so that 1000 flies (about 5 gm. weight) would yield only 0.25 μg. of a metabolite produced in a 10% yield, and this would give but one point on a graph showing the time course of production. Only with the recent radioactive preparations of high specific activity (Chapter 10) plus good separatory techniques can one make effective studies of this kind. However, where phosphates or phosphonates are used rather than phosphorothionates, etc., the fraction extractable by chloroform (or other organic solvents) represents only one compound—the parent material; in these cases, a very simple procedure gives a very precise picture of the most important material. Thus, Arthur and Casida[1] studied the degradation of Dipterex and DDVP in the housefly, and found that in 4 hours about 60% of the former and 50% of the latter had been degraded.

HYDROLYSIS

Malathion hydrolysis was first studied in cockroaches by March *et al.*[62] who found in the gut, 2 hours after poisoning, only one polar metabolite which was separable from malathion using paper chromatography. Later studies by Krueger and O'Brien[47] using column chromatography showed, in the whole cockroach, ten chloroform-unextractable metabolites of which six were identified. Table 7.4 gives data for the unextractable metabolites in the American and German cockroach and the housefly. In the cockroaches, carboxyesterase action accounted for up to a half of the degradation; in the housefly, carboxyesterases were far less important.

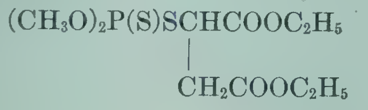
A most important paper by Plapp and Casida, in 1958, has given data on the exact place of cleavage of six organophosphates by the American cockroach (Table 7.5). In these cases, only phosphatase action occurred, so the picture is less complex than for malathion; of the six possible products of combined oxidation and phosphatase action, only one was always absent: dialkyl phosphorodithioate, $(RO)_2P(S)SH$. This may be due to the possible lability of such a compound—it might be oxidized to $(RO)_2P(S)OH$ as fast as it was produced. It is interesting that the cleavage of the P—O—alkyl group is very small, except for methyl parathion. It will be recalled that this cleavage was of considerably greater importance in the mammal

Table 7.4. Hydrolysis Products of Malathion in Insects Treated at Half the LD_{50} [a, b]

Insect	Hours after treatment	Dose mg./kg.	Per cent of total hydrolysis products as								
			Phosphatase products					Carboxyesterase products			Unknowns[c]
			$(OH)_2P(O)OH$	$(MeO)_2(PO)OH$	$(MeO)_2P(S)OH$	$(MeO)_2P(S)SH$	Total	Monoacid	Diacid	Total	Total
American cockroach	0.5	4	5	6	12	6	29	42	8	50	21
	24	4	4	19	27	0	50	44	0	44	6
German cockroach	0.5	60	0	8	22	14	44	43	0	43	13
	24	60	4	18	25	0	47	17	19	36	17
Housefly	0.5	15	0	7	36	6	49	27	0	27	24
	24	15	5	20	42	0	67	10	2	12	21

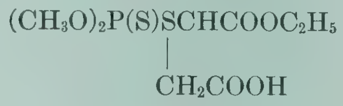
[a] Data selected and rounded off from Krueger and O'Brien.[47] *Treatment:* topical.

[b] Malathion:



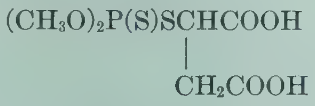
$$(CH_3O)_2P(S)SCHCOOC_2H_5 \quad | \quad CH_2COOC_2H_5$$

Monoacid of malathion:



$$(CH_3O)_2P(S)SCHCOOC_2H_5 \quad | \quad CH_2COOH$$

Diacid of malathion:



$$(CH_3O)_2P(S)SCHCOOH \quad | \quad CH_2COOH$$

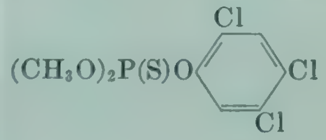
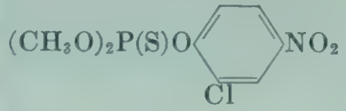
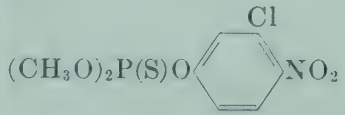
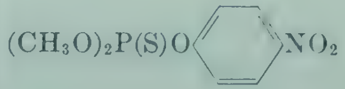
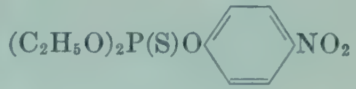
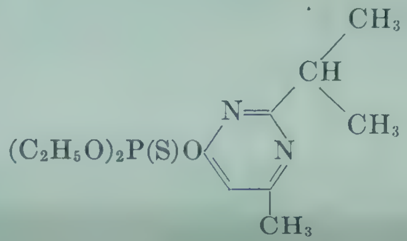
[c] Original data separates these into four compounds.

(pp. 227, 231). Clearly, the major product in the cockroach is dialkyl phosphorothioate, $(RO)_2P(S)OH$. This can be derived from these particular compounds in only one way: by hydrolysis of the P—O—X group before oxidation of P═S to P═O has occurred. We may also conclude that the oxidation of $(RO)_2P(S)OH$ to $(RO)_2P(O)OH$ is not very extensive in the American cockroach, and therefore that the especially high level of $(RO)_2P(O)OH$ to which Diazinon gives rise is due to hydrolysis of diazoxon produced by the oxidation of P═S to P═O.

In another study,[93] these authors showed that in houseflies hydrolysis of ronnel (formula in Table 7.5) produced dimethyl phosphorothioate, $(CH_3O)_2P(S)OH$, almost exclusively (89% of total hydrolysis products in 1 hour) with a little (8%) cleavage of the CH_3—O—P.

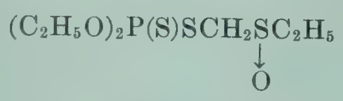
Bowman and Casida[14] studied the metabolism of Thimet products by

TABLE 7.5. HYDROLYSIS OF SIX ORGANOPHOSPHATES IN THE AMERICAN COCKROACH[a]

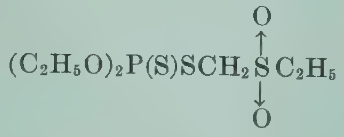
Compound	(RO) P(S)OX	Dose mg./kg.	Per cent of total hydrolysis products as:			
			$(RO)_2P(O)OH$	$(RO)_2P(S)OH$	$\begin{matrix}RO\\HO\end{matrix}\!>P(O)OX$	$\begin{matrix}RO\\HO\end{matrix}\!>P(S)OX$
Ronnel		20	16	76	1	7
Dicapthon		20	3	93	trace	4
Chlorthion		20	2	97	trace	1
Methyl parathion		4	27	47	3	23
Parathion		20	28	67	3	2
Diazinon		20	41	59	trace	trace

the southern armyworm *Prodenia eridania.* Thimet, $(C_2H_5O)_2P(S)SCH_2SC_2H_5$, was applied to plants on which the insects were allowed to feed. From the differences between the metabolites present in the plants and those present in the insect bodies and feces, certain conclusions may be drawn.

(*a*) The principal material consumed by the insects was the sulfoxide and/or sulfone derivatives of Thimet (these were not separable chromatographically).


$(C_2H_5O)_2P(S)SCH_2\underset{\underset{O}{\downarrow}}{S}C_2H_5$

Sulfoxide


$(C_2H_5O)_2P(S)SCH_2\overset{\overset{O}{\uparrow}}{\underset{\underset{O}{\downarrow}}{S}}C_2H_5$

Sulfone

(*b*) About 96% of these derivatives were altered by the insect within 1 day; oxidation to the phosphate analogs was substantial (about 17%), but degradation accounted for most of this alteration: diethyl phosphate, $(C_2H_5O)_2P(O)OH$, was the principal product (41% of total), and next was diethyl phosphorodithioate, $(C_2H_5O)_2P(S)SH$, (7% of total).

(*c*) The composition of the metabolites in the feces was extraordinarily like that in the whole insect, even after 12 days. There was, therefore, no evidence that the hydrolysis products could be excreted more readily than the oxidation products. It will be recalled that in the case of TEPP, by contrast, the American cockroach excreted only hydrolysis products.[33]

OXIDATION

Plapp and Casida[94] mentioned in passing that with the six compounds of Table 7.5 they found the P=O analogs to be formed *in vivo* in the American cockroach. Now such an oxidation has, of course, been assumed to occur in insects for many years, since: (*a*) pure phosphorothionates are poor anticholinesterases *in vitro*, yet produce strong cholinesterase inhibition *in vivo*; (*b*) phosphorothionates and their corresponding phosphates have fairly similar LD_{50}'s to insects;[74] and (*c*) such oxidations occur with insect tissues *in vitro* (Chapter 4).

More detailed experiments upon the oxidation of P=S to P=O in insects were carried out by Krueger *et al.*[47, 48] with malathion, Diazinon, dimethoate, acethion, and parathion. The time course of this oxidation was traced in the housefly and American cockroach, and for malathion, only, in the German cockroach also. The matter will be discussed in detail in Chapter 9 in relation to its significance for comparative toxicology.

Systox metabolism in the cockroach was studied by March *et al.*:[63] In gut they found two oxidation products from the thiono isomer and one oxidation product from the thiolo isomer; identification of these products was not possible. Menn[70] has shown that in the German cockroach, milkweed bug, and *Rhodnius* bug the principal oxidation was of the mercapto sulfur to produce either the sulfoxide or the sulfone (which were not separable).

DEHYDROCHLORINATION

The metabolism of Dipterex in insects has received special attention because of the possibility that *in vivo* it rearranges with dehydrochlorina-

$$\underset{\text{Dipterex}}{(CH_3O)_2P(O)CH(OH)CCl_3} \xrightarrow{-HCl} \underset{\text{DDVP}}{(CH_3O)_2P(O)OCH{=}CCl_2}$$

tion to DDVP, which would then be the actual toxicant as it is a somewhat better anticholinesterase and a much more toxic compound than Dipterex itself. This dehydrochlorination occurs very easily in alkali (page 67) Arthur and Casida[1] consider that the reaction does not occur *in vivo* since (*a*) no chloroform-extractable compound except Dipterex was present in poisoned flies or in American or Madeira cockroaches as judged by chromatography, changes in solubility, antiesterase activity, or permanganate-reducing characteristics (specific data were not given); (*b*) cockroach guts *in vitro* did not affect the anticholinesterase activity of Dipterex (but then the difference between the anticholinesterase activity of Dipterex and DDVP is very small compared to the thousandfold differences used in studying phosphorothionate activation); and (*c*) houseflies degraded Dipterex more rapidly than DDVP.

Metcalf *et al.*[73] on the contrary consider that all the biological effects of Dipterex are due to DDVP, as discussed on page 224.

Resistance

OCCURRENCE

Whereas resistance to DDT has been a serious problem for almost a decade, reports of resistance to organophosphates have been relatively sparse and only in the last few years has the seriousness of the problem been established. Table 7.6 gives an indication of the situation as of 1959; it is not intended to be exhaustive. The table shows that the extent of resistance is usually considerably less than for chlorinated hydrocarbons, even with prolonged exposure.

March[61] has studied the induction of resistance in laboratory strains of houseflies by four organophosphates (Fig. 7.9). The technique used was to treat the successive generations with enough insecticide to permit 20–40% survival. The results are alarming in some ways. Although as Table 7.6 shows, field resistance is commonly of a low order of magnitude compared

TABLE 7.6. OCCURRENCE OF RESISTANCE[a]

Insect	Compound	Resistance factor	Number of generations	Location	Year reported	Reference
Housefly (adult)	Paraoxon	11	40	U.S.A.	1955	72
	DFP	10	70	U.S.A.	1955	72
	Parathion	18	148	U.S.A.	1955	72
	Diazinon	16	14	Holland	1956	66
	Malathion	100	61	California	1959	61
	Chlorthion	500	52	California	1959	61
	Diazinon	20	52	California	1959	61
	Parathion	10	205	California	1959	61
	Parathion	5	3 years	Denmark	1956	45
	Diazinon	5	3 years	Denmark	1956	45
	Co-ral	40	3 years	Denmark	1956	45
	Malathion	5–20	4 years	U.S.A.	1957	53
	Dipterex	5–20	4 years	U.S.A.	1957	53
	Diazinon	60	4 years	Italy	1957	101
	Malathion	23–37	—	Florida	1957	49
	Malathion	2–4	2 years	Georgia, U.S.A.	1957	46
Housefly (larvae)	Diazinon	30	—	Israel	1956	71
Chrysomia putoria (a fly)	Diazinon	300	2 years	Belgian Congo	1958	13
Culex tarsalis mosquito (adult)	Malathion	93–100	2.5 years	California	1957	35
Culex tarsalis (larvae)	Malathion	21–33	2.5 years	California	1957	35
Spotted alfalfa aphid	Parathion	4	—	California	1958	117
Aedes nigromaculis mosquito (larvae)	Parathion	3	5–6 years	California	1959	50

[a] Table expanded from Pal.[91]

to that found with chlorinated hydrocarbons, the results of Fig. 7.9 indicate that severe resistance may be achieved. Chlorthion has the worst potential: a thousandfold resistance was obtained after 59 generations of selection. A flattening of the curve at some maximum peculiar to each compound is evident: parathion resistance was no more severe after 205 generations than after 48 generations of selection; the maximum resistance factors appear to be about 1000 for Chlorthion, 100 for malathion, 20 for Diazinon, and 10 for parathion. This does not prove that each has its own resistance mechanism: for instance, if it were a decrease in nerve cord permeability that caused resistance, this would depress the toxicity of labile

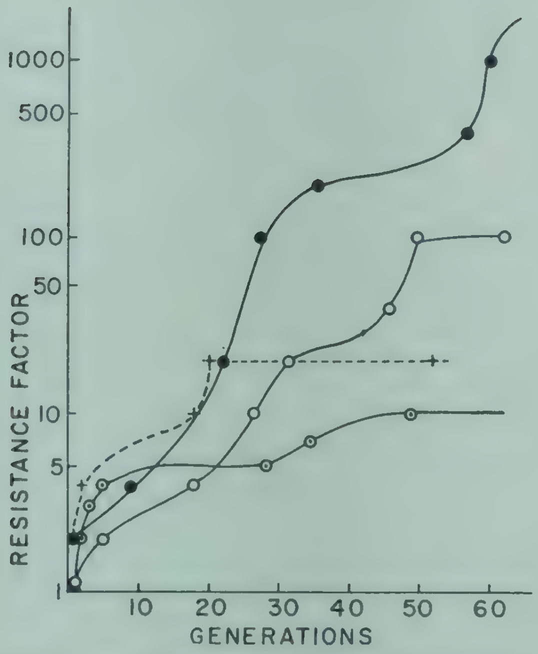


FIG. 7.9. Development of resistance to organophosphates by houseflies. The indicated insecticides were used to induce resistance and to test for it. Data plotted from March.[61]

● = Chlorthion; ○ = malathion; × = Diazinon; ⊙ = parathion.

poisons much more than stable poisons, since the labile ones would be degraded "while waiting to get into the nerve."

March also studied the decline of resistance that occurred when exposure and consequent selection was stopped. With parathion, resistance declined rapidly and promptly for seven generations and then remained steady at a low (twofold) level for the further 18 generations studied. With Chlorthion and malathion the decline continued steadily and rapidly throughout the 25 generations studied, e.g., with malathion, the LD_{50} in μg./gm. fell from >100 to 0.48.

CROSS RESISTANCE

Fortunately, repeated exposure to chlorinated hydrocarbon insecticides induces little resistance to organophosphates.[11, 32, 60, 61] Unhappily, repeated exposure to organophosphates induces enormous resistance to chlorinated hydrocarbons. March,[61] for example, found that a strain of houseflies selected for 41 generations with parathion acquired only 7-fold resistance to parathion, but over 3000-fold resistance to DDT, 70-fold to methoxychlor, 1.6-fold to Prolan, 120-fold to lindane, and over 3000-fold to dieldrin (topical doses throughout). Similarly, a Diazinon selected strain[66] was only 10 times resistant to Diazinon, but 3000 times resistant to toxaphene. Furthermore, when the organophosphate exposure is stopped, the tolerance

TABLE 7.7. RESISTANCE OF CITRUS RED MITE TO ORGANOPHOSPHATE SPRAYS[a]

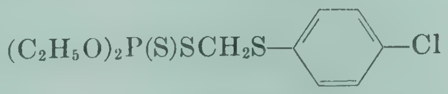
Compound	Times increase in LC_{50}	Times increase in LC_{95}
Trithion	15,000	30,000
Iso-Systox	8,570	2,900
Methyl Systox	8,000	4,000
Schradan	2,000	700
EPN	1,400	1,600
Parathion	883	2,330
Phosdrin	350	600
Pyrazoxon	300	50
Systox	266	192
Tetram	111	91
Delnav	100	18
Disyston	25	80
Diazinon	12	333
Malathion	8	9
Ethion	8	6

[a] Results of Jeppson *et al.*[43] Resistance was induced in the field with Systox.

to organophosphates rapidly decreases in a few generations, yet the DDT resistance is maintained.[61]

It is difficult to imagine an explanation for such phenomena. The fact that the cross resistance is not reciprocal seems to preclude simple genetic linkage of physiologically independent factors. The possibility that some nonspecific protection (such as cuticular thickening, or reduction of penetrability of the nerve sheath) is induced by the organophosphates would be an obvious explanation if it were not for the fact that recession of one resistance occurs without recession of the other. It must be that several factors contribute to resistance: a nonspecific factor which by itself is useful only in protecting against chlorinated hydrocarbons; and one or more group- or compound-specific factors, which recede on withdrawing "insecticide pressure."

An examination of cross resistance within the organophosphates also reveals a complex situation. Table 7.7 shows that when mites developed resistance in the field to Systox, an astonishing range of resistances accompanied the phenomenon. Their resistance to Trithion was fifteen thou-



Trithion

$(C_2H_5O)_2P(S)SCH_2SP(S)(OC_2H_5)_2$

Ethion

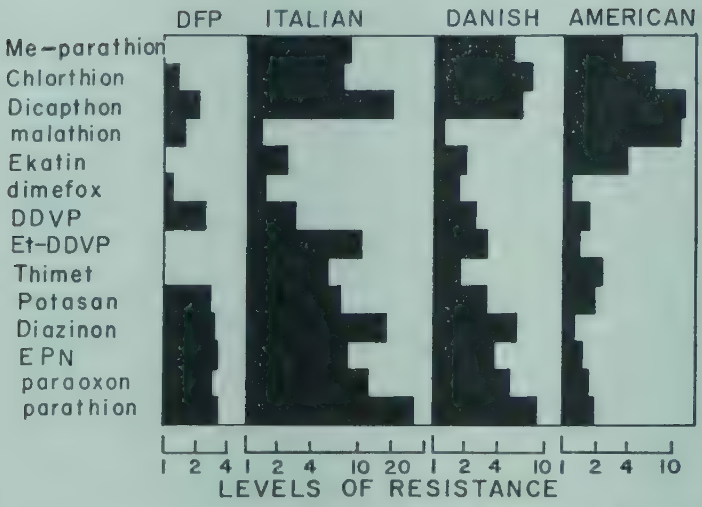


Fig. 7.10. "Resistance spectra" of four housefly strains. Level of resistance = (LD_{50} resistant)/(LD_{50} susceptible). From Busvine.[16]

sandfold, to ethion only eightfold. The table also shows that, in general, the resistance at the LD_{95} was substantially the same as that at the LD_{50}, thus introducing a welcome simplification into the interpretation.

An extensive study by Busvine[16] of cross resistance in houseflies is summarized in part in Fig. 7.10. Some trends may be seen: resistance to dimefox was always low, and resistance to dicapthon was usually high; qualitatively, the silhouette of the "resistance spectra" show similarities, but differences are also in excellent supply. It is interesting to note that the insecticide used for inducing resistance was in no case the insecticide to which greatest resistance was observed. This was also true in some of the cases studied by March:[61] a parathion-selected strain was 6 times resistant to parathion, 74 times to malathion; a Diazinon selected strain was 9 times resistant to Diazinon, 57 times to malathion. However, in March's study, selection by malathion and Chlorthion induced most resistance towards the selecting compound. Finally, there are a number of cases in which selection by compound A has little effect upon the susceptibility to compound B, for example, when A = Diazinon and B = malathion, A = parathion and B = malathion, A = malathion and B = dimefox (Figure 7.10); and A = malathion and B = Dipterex.[49]

Another extensive study of cross resistance was made for houseflies by Forgash and Hansens,[34] using a strain selected by Diazinon treatment from flies which had already developed Diazinon resistance in the field. Table 7.8 shows the large increases in resistance observed with every class of insecticide studied. In this case, the selector organophosphate was the most resisted organophosphate. However, organophosphate resistance was dwarfed by chlorinated hydrocarbon resistance, for instance, DDT had its

TABLE 7.8. RESISTANCE IN A DIAZINON SELECTED STRAIN OF HOUSEFLIES[a, b]

Class	Compound	Times increase in LD_{50}	Times increase in LD_{95}
Organophosphates	Diazinon	38	42
	Malathion	5	6
	Parathion	16	22
	Ronnel	18	17
	Chlorthion	23	12
Pyrethrins	Pyrethrin	4	3
	Allethrin	5	3
Carbamates	Isolan	14	5
	Pyrolan	10	12
	Sevin	>37	—
DDT-like	DDT	>6400	—
	TDE (=DDD)	>1290	—
	Methoxychlor	>3500	—
	Dilan	10	8
Other chlorinated hydrocarbons	Chlordan	396	37000
	Dieldrin	220	2060
	Lindane	254	7050
Thiocyanate	Lethane 384	2	2

[a] Data of Forgash and Hansens.[34]

[b] *Note*: The comparison was between a susceptible laboratory strain and a Diazinon-resistant field strain which had been further selected by Diazinon in the laboratory. There was, therefore, no guarantee that the field strain had not been in contact with DDT or other insecticides.

LD_{50} increased more than 6400 times. Unfortunately, one cannot evaluate the importance in this study of a prior exposure of the population, several years earlier, to the carbamate Pyrolan and to methoxychlor.

Oppenoorth[90] has examined cross-resistance patterns of 13 organophosphates in seven housefly strains. Of these strains, one was unselected, another had been selected with the chlorinated hydrocarbon lindane, one had been selected with parathion, one with malathion, and two with Diazinon. Substantially similar results were found both with topical and contact applications. On the whole, the cross-resistance pattern was similar in all cases, unlike the situation reported by Busvine. Thus, the decreasing order of LD_{50}'s for virtually all strains was malathion, Guthion, Diazinon, methyl paraoxon, methyl parathion, parathion, and DDVP. The responses to Co-ral and its oxygen analog were more heterogeneous. Oppenoorth felt that "each strain has its own pattern of resistance," but, in fact, the similarities were more apparent than the differences.

In several cases reported above, massive resistance to insecticides were found, particularly for chlorinated hydrocarbons. Such findings do not necessarily mean that the *intrinsic* resistance was massive; van Asperen[5] has pointed out that the data are usually from topical applications, and at very high doses penetration may become a limiting factor. This consideration is supported by the results of Bridges[15] who found that 24 hours after the topical application of allethrin, 95 % of a low dose (1 μg.) was absorbed, but only 47 % of a higher dose (12 μg.) was absorbed. For similar reasons, results obtained by exposure to treated surfaces are an even poorer index of intrinsic resistance, for one has to consider limitations in the availability of the insecticide to the insect and limitations in the ability of the insect to pick up the available insecticide, as well as the above limitation on penetration.

Mechanisms of Resistance

The following factors have been studied in an attempt to explain resistance: cuticular penetration, rates of degradation, storage, and cholinesterase susceptibility. The two methods used have been those with homogenates *in vitro*, and those with intact insects. The homogenate method offers the advantage of being able to work with large quantities of inhibitor, so that one may use anticholinesterase assays to follow appearance or disappearance of phosphate, or use radioactive material of low specific activity. With intact insects only a little compound may be used, since the insects must live, but presumably the results are more meaningful, for we are primarily interested in events *in vivo*.

(*1*) *In Vitro Methods*

March[61] found that a sliced housefly preparation from malathion resistant flies degraded malaoxon (the activation product) 2–3 times faster than a similar preparation from susceptible flies. Van Asperen and Oppenoorth[8] studied the degradation of paraoxon and diazoxon (the phosphate analog of Diazinon) by housefly homogenates and found that degradation was substantially faster in resistant (D) than in susceptible (S) flies, and particularly rapid in a particularly resistant strain (C). They pointed out that the amounts degraded were fairly small compared to the LD_{50}'s: thus, with diazoxon the LD_{50} for the C strain was 0.5 μg. per fly, yet degradation *in vitro* was only 0.036 μg. per hour per fly. The corresponding figures for the D strain were 0.07 and 0.01. They therefore suggested that "it seems likely that its function is to prevent fatal concentrations from building up near the site of toxic action" and point out that this presupposes an uneven distribution in the body. However, as Oppenoorth has pointed out elsewhere,[90] the variety of cross-resistance patterns suggests that there are a variety of resistance mechanisms. It seems likely, therefore, that a given resistant strain may show various resistance mechanisms, and it seems inadvisable to attribute resistance to any one mechanism until it can be clearly shown to be adequate to account for the observed resistance.

Van Asperen and Oppenoorth[7] had previously shown a curious but consistent difference between susceptible and resistant houseflies: aliesterase* was consistently lower in the four resistant than in the six susceptible strains. With ethyl butyrate as substrate, the average difference was sixfold. Later,[8] they quoted unpublished work of Nguy and Busvine and of Oppenoorth showing that a single gene mutation converts part of the aliesterase into a phosphatase. If this suggestion is correct, it explains, neatly, both the raised phosphatase levels and the lowered aliesterase levels.

There appears to be general agreement[7, 59, 61, 89] that cholinesterase from susceptible and from resistant insects is equally sensitive to inhibition by phosphates *in vitro*.

(2) *In Vivo Methods*

The cases of cross resistance quoted above show numerous examples of resistance both to phosphorothionates and to their corresponding phosphates. Therefore it is unlikely that resistance can be caused by low levels of activating enzymes.

Direct measurements on two housefly strains, one susceptible and the other 40 times resistant to Diazinon, showed only a 7% difference in the rate of Diazinon penetration, regardless of dose.[48] For parathion, resistant houseflies showed marked resistance even to injected doses, so that penetration differences could not account for resistance.[89] However, they were susceptible to injected paraoxon, although resistant to topically applied paraoxon. This suggests that the resistance is caused by rapid degradation, which has no opportunity to show its action if paraoxon is delivered suddenly into the body (cf. the "opportunity factor," page 323).

Various attempts have been made to find differences in degradation rates *in vivo*. Lord and Solly[59] found that a susceptible and a two times resistant strain of houseflies degraded topically applied paraoxon, 0.1 μg. per fly, at precisely the same rate. Substantially similar conclusions were reached as a result of studies with radioactive methyl parathion, malathion, and Diazinon.[48, 69] These conclusions are at variance with those of Oppenoorth[89] who found that paraoxon was degraded more rapidly after its application to resistant houseflies, and furthermore, that after parathion injection there was a considerable accumulation of paraoxon in susceptible flies but none in very resistant flies. However, his method was not very satisfactory: he homogenized the flies at various times, incubated the homogenates, and used the resultant inhibitions of endogenous cholinesterase as an index of paraoxon. This method does not distinguish between inhibition *in vivo* prior to homogenization and subsequent *in vitro* inhibition. Mengle *et al.*[69] used the more satisfactory procedure of adding further cholinesterase after homogenizing and could then find no difference between susceptible and resistant houseflies.

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* Originally defined as an enzyme hydrolyzing short-chain aliphatic esters. However, it is not a specific enzyme, and hydrolyzes aromatic esters too. (See page 206.)

Darrow and Plapp[30] found no difference in the rate of degradation of malathion by resistant and susceptible larvae of the mosquito *Culex tarsalis*, when immersed in a 0.05 p.p.m. solution of malathion: both degraded about 34 % of the malathion in 48 hours. If a difference in the amount of a specific malaoxon-hydrolyzing enzyme was responsible, this technique, perhaps, would not distinguish the difference, e.g., if only a little malathion was activated to malaoxon. Rasmuson and Holmstedt[98] used a rather indirect and unsatisfactory technique to study paraoxon hydrolysis in homogenates of parathion-resistant *Drosophila*, and found no evidence of a difference from the susceptible strain (they added the organophosphate and acetylcholine, and followed the time course of inhibition of acetylcholine hydrolysis).

With houseflies 40 times resistant to Diazinon, Krueger *et al.*[48] found only a small difference from a susceptible strain in penetration or metabolism of topically applied Diazinon. The insecticide was labeled with radioactive phosphorus, and applied at 4 and at 30 μg./gm. There was about a 7 % faster penetration in the susceptible flies, and they produced about twice as much diazoxon. Finally, the combined levels of Diazinon and diazoxon were (at maximum) about three times higher in the susceptible flies, suggesting that net removal of Diazinon was somewhat faster in the resistant flies. Resistance could not be reliably attributed to these differences.

Detailed studies by Mengle *et al.*[69] on the metabolism of radioactive Diazinon, malathion, and methyl parathion by resistant and susceptible houseflies have shown relatively small differences in the levels of parent compound, phosphate analogs, and degradation products at various times; in no case have such differences warranted attribution of resistance to them. Yet it is the writer's opinion that only such studies can show whether resistance is indeed due to differences of activation, degradation, or penetration.

In summary of our meager knowledge on resistance, the only hopeful clue is that degradation, usually of the phosphate analog, by whole or homogenized insects, appears, in some cases, to be somewhat greater in resistant than in susceptible strains. The differences and the amounts involved are not large enough to offer a convincing hypothesis of the resistance mechanism. But if it can be demonstrated that these differences actually lead to marked differences of actual toxicants at localized sites, degradative effects could be considered to play an important role. We should expect several kinds of organophosphate resistance mechanisms, although, conceivably, these kinds may simply involve different phosphatases of restricted specificity. The remarkable induction of chlorinated hydrocarbon resistance which follows selection by organophosphates suggests that numerous changes occur during the development of organophosphate resistance, some of which may make little direct contribution to the organophosphate resistance. This should make us the more wary of attributing resistance to any one property which is found to differ in susceptible and resistant strains.

Antagonism and Synergism

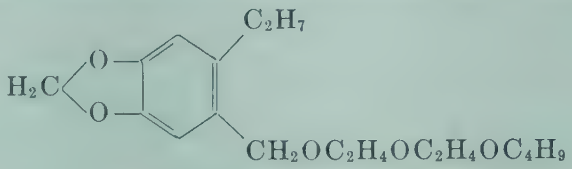
ANTAGONISM

Chapter 6 has described how many oximes are effective in antagonizing the toxicity of organophosphates to mammals. In the insect, little or no protection is found. Thus, for the housefly, Winteringham *et al.*[123] showed that 2-PAM (pyridine-2-aldoxime methiodide) had no effect upon the LD_{50} of DFP. Mengle and O'Brien[68] found that injected 2-PAM increased the LD_{50} of DFP 1.5 times, but neither 2-PAM nor pyridine-2-aldoxime dodecaiodide nor diisonitrosoacetone had any effect upon the LD_{50} of parathion, methyl parathion, paraoxon, malathion, or TEPP. Both groups of workers noted, however, that 2-PAM could delay the early paralysis caused by organophosphates.

It seems probable that the very poor effectiveness of these agents is attributable to their ionized state, which keeps them from penetrating the nervous system. To be more precise, the fact that these compounds are effective in mammals may be due to the mammals' having two relatively ion-available areas: the autonomic ganglia and the neuromuscular junction. The insect appears to have no important cholinergic ion-available sites.

Another possible antagonist to organophosphate poisoning is SKF 525A (page 209). This was found to be ineffective in protecting flies from Co-ral, Diazinon, Guthion, ronnel, Dowco 109, or dimethoate, or in protecting American cockroaches from ronnel or Guthion.[81]

An antagonism between piperonyl butoxide and malathion has been noted by Rai *et al.*,[95] e.g., the LD_{50} of malathion to the housefly was raised from 26 to 39 μg./gm. by a tenfold excess of piperonyl butoxide. Rai and Roan[96] showed that piperonyl butoxide, *in vivo* or *in vitro*, had a small protective effect against inhibition or fly cholinesterase by malathion *in vitro*. The significance of this is somewhat doubtful since malathion itself is a poor anticholinesterase, and it is malaoxon which is the actual anticholinesterase in malathion poisoning. Later, these authors[97] studied the role of the locus of application and the time between application of malathion and piperonyl butoxide. Both factors were of importance in determining the toxicity of the combination.



Piperonyl butoxide

SYNERGISM

In mammals a number of pairs of organophosphates, when administered together, show a greater toxicity than expected from the sum of their individual actions. This is particularly true if either EPN or triorthocresyl phosphate (TCP) is one of the pair, and if the other is an organophosphate containing a carboxyester or carboxyamide grouping. This is attributable to the EPN or TCP inhibiting the detoxification which normally occurs by hydrolysis of the carboxyester or carboxyamide (pages 212).

Seume and O'Brien[105] pointed out that in (for instance) carboxyester-containing organophosphates, the importance of carboxyester hydrolysis in detoxification is great in the mouse, less in the American cockroach, and still less in the housefly. It should follow that the extent of potentiation should fall off in the same order. Their data did not permit precise evaluation of the extent of synergism; however, it was clearly shown that EPN synergized dimethoate and malathion in the American cockroach and housefly, and this synergism was less extensive than in the mouse. As in the mouse (but to a smaller extent) Dowco 109 was synergized by EPN, an unexpected phenomenon discussed on page 216.

Synergism between piperonyl butoxide and either Diazinon or Dipterex has been reported qualitatively by Rai and Roan.[96]

Synergism of seven organophosphates by 19 nonphosphorus compounds (many of them pyrethrum synergists) was reported by Hoffman *et al.*, using DDT-resistant houseflies. Table 7.9 shows the results, which are complex in that some synergists are best for one organophosphate, others for another; thus, piperonyl butoxide was excellent with Co-ral, useless with malathion. The case of piperonyl butoxide synergism of Co-ral or its phosphate analog "coroxon" was studied further by Monroe and Robbins.[78] They found that piperonyl butoxide *in vitro* had little effect upon the *in*

TABLE 7.9. ORGANOPHOSPHATE SYNERGISTS FOR HOUSEFLIES[a]

Number	Synergist	Malathion 5:25[b]	EPN 1:10	Methyl-parathion 1:10	Co-ral 5:25	Compound 21/200 5:25	Potasan 5:25	Diazinon 1:10
	None	3	22	51	20	4	18	23
1.	*N*-Isobutylundecylenamide	70	98	97	87	6	100	75
2.	Chrysanthemumic acid, alpha-propyl piperonyl ester	7	73	89	100	86	100	81
3.	Chrysanthemumic acid, alpha-ethyl piperonyl ester	7	90	84	100	64	100	59
4.	Chrysanthemumic acid, alpha-isopropyl piperonyl ester	9	54	84	87	31	90	77
5.	*m*-Dioxane, 5-butyl-5-ethyl-2-(3,4-methylenedioxyphenyl)	16	79	82	100	75	100	68
6.	Piperonyl cyclonene	0	2	16	64	10	0	13
7.	Sulfoxide	0	58	87	97	86	94	94
8.	Piperonyl butoxide	4	99	98 (0.5:5)	100	97	100	96
9.	*n*-Propyl isomer	0	6	3	76	36	30	32
10.	Piperonylidenemalonic acid, diethyl ester	3	53	58	80	49	71	—
11.	Hexahydrophthalic acid, di-*n*-butyl ester	78	90	87	75	40	100	72
12.	Phthalic acid, methyl-*n*-hexyl ester	56	96	97	98	70	90	—
13.	*N*,*N*-Diamylsuccinamic acid, propyl ester	35	90	95	82	57	88	—
14.	*N*,*N*-Dipropyl succinamic cid, ethyl ester	67	99	68	88	26	88	—
15.	*N*,*N*-Diethylsuccinamic acid	19	18	27	45	0	53	—
16.	*N*,*N*-Diisopropyl succinamic acid, ethyl ester	32	98	76	80	11	93	—
17.	*N*,*N*-Diethyl glutaramic acid, ethyl ester	83	80	67	78	63	97	—
18.	*N*,*N*-Dipropyl glutaramic acid, methyl ester	65	97	95	93	69	82	—
19.	*N*-(2-Ethylhexyl)bicycloheptenedicarboximide	33	92	88	88	67	89	—

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[a] Data of Hoffman *et al.*[37]

[b] Per cent mortality of resistant houseflies 24 hours after a 10-minute exposure to residues of organic phosphorus compounds alone and in combination with the candidate synergists; 2 to 5 replications. The ratios in the column headings indicate the ratio of toxicant to synergist in milligrams per square foot.

vitro anticholinesterase action of Co-ral or coroxon. But when piperonyl butoxide was applied *in vivo* along with Co-ral, there was substantially more cholinesterase inhibition than with Co-ral alone, e.g., 57 % inhibition in place of 21 % for Co-ral alone. There was no evidence as to the mechanism of this phenomenon.

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CHAPTER 8

Effects in Plants

Systemic Activity*

Systemic activity may be defined as the ability of a material to be translocated within the plant. There are, however, a number of cases of insecticides which can be translocated to a very limited extent, for instance, from one surface of a leaf to the other, and it is not customary to call such insecticides "systemic": the term "Tiefenwirkung" (deep action) has been used to describe this limited translocation.[114] Bennett[7] has defined systemic action more narrowly, as involving absorption and translocation to other parts of the plant in such a way as to render those parts insecticidal. This represents a less drastic definition than an earlier one of his[6] which required that the material be translocated to all parts of the plant, making them insecticidal.

A further classification of systemic insecticides has been proposed by Ripper:[91, 92] (*a*) stable: compounds not metabolized in the plant, (*b*) endolytic: compounds which are degraded and not activated in the plant, so that the only toxic substance (or 98% of the toxic substances) is the parent compound, and (*c*) endometatoxic: compounds subject to activation by the plant as well as degradation, so that insecticidal materials other than the parent compound are present in the plant. These factors will be discussed more fully in the section on metabolism. In this section only the translocation of toxic materials will be considered.

The first observation of systemic action with organic insecticides was made in 1935 by Schrader and Kukenthal, using condensation products of fluoroethanol with aldehydes. In 1941, Schrader and Kukenthal made the first two systemic organophosphates, dimefox and schradan, and demonstrated their systemic activity (Geary[45]).

$[(CH_3)_2N]_2P(O)OP(O)[(CH_3)_2N]_2$ Schradan

$[(CH_3)_2N]_2P(O)F$ Dimefox

Nowadays about 13 systemic insecticides are in common use[72] of which perhaps the ones of greatest importance, in order, are Systox, meta-Systox, Phosdrin, schradan, Thimet, and dimethoate.

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* Three excellent reviews on this topic were published in 1957 by Bennett,[7] Metcalf,[71] and Ripper,[92] and consequently this section will not attempt to give a full account.

Intensive studies on the systemic action of organophosphates were carried out as the result of proposals by Martin, in 1947 (see Ripper *et al.*[93]). The early work was on schradan and dimefox, the test of systemic action being the appearance of toxicity usually to aphids in parts of the plant other than the place of application (presumably, this procedure accounts for the form of Bennett's definition of systemic action, above). In 1949, Bennett[6] showed that the above two compounds were translocated in cabbage plants growing in treated soil, or in willow shoots grown in treated nutrient solution. He noted that unless precautions were taken, a direct fumigant action of the compounds could show itself.

David,[27] in 1951, examined radioactive schradan translocation in strawberries and various vegetable plants following root or leaf application. Little translocation from young to old leaves occurred, but considerable translocation into leaves developed subsequent to treatment was observed: it seemed that schradan moved passively with the flow of other materials. Consequently, downward translocation was slight except when perennials were building up root reserves. Ripper *et al.*[93] reported that following local application of schradan to hops or sugar beets, translocation upwards but now downwards occurred. However, later work by these authors[94] with schradan on chrysanthemums showed translocation to be equally excellent in both directions, and Zeid and Cutkomp[121] found, for young beans, that schradan and paraoxon were translocated far better downwards than upwards. In flowering beans, by contrast, no downward translocation could be observed. In apple seedlings, downward translocation of schradan was clearly seen but was far less extensive than upward translocation.[110] On implantation of dimefox into the trunks of mature cacao trees, only upward translocation was observed.[59] Perhaps the most convincing observation was that in sprayed groundnut plants, schradan (or its metabolites) moved persistently downward into the growing nuts, and from there out to the soil, so that by 25 days after spraying 28% of the dose was in the "soil-plus-roots" fraction.[107]

These results seem somewhat discordant, but, in fact, are not incompatible. In every case where radioactive tracers were used, some downward translocation was observed, but often in amounts which the more frequently used bioassays could not detect. It seems safe to say that, in general, the systemics, wherever applied, travel in the direction of the nutrients: in most cases examined, it has happened that the nutrients flow principally upwards. It is surely significant that in the single case of the groundnut, where extensive downward flow of nutrients occurred, downward translocation of the insecticide was very marked.

Light has been shown to be of importance in translocation following leaf application of schradan to beans[110] and Systox to lemon cuttings.[117] For instance, with beans sprayed locally with schradan, in daylight 3% of the absorbed dose was translocated to other parts of the plant, in the dark 1% was translocated.

The phosphorus available to the plant may be important in dictating the extent of organophosphate uptake. Casida *et al.*,[20] working with pea plants found that schradan was taken up by the root twice as well from a phosphorus deficient medium than from a complete nutrient medium. However, in a series of seven soil types, uptake was not correlated with content of available phosphorus; instead the schradan was taken up best from sands, next from loam, worst from clay. Probably the matter of retention of the insecticide by the soil outweighed that of available phosphorus.

The question of the pathway for translocation after foliar spraying has been examined for Systox in cuttings of lemon[117] and *Pelargonium*,[111] and for schradan in apple seedlings,[110] by ringing experiments. In all cases, it was shown that the phloem was the major route, for ringing severely reduced translocation. However, a delayed, slight translocation in the ringed plants showed that limited movement through xylem could occur. A second piece of evidence that the phloem was the main route was that the translocation rate (of schradan in bush beans) was of similar magnitude to that observed for various organic materials through phloem.[118]

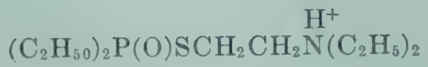
The route of translocation after root application is stated by Tietz[111] and Ripper[92] to be by the xylem. However, there seems little evidence to support this; in fact defoliation of cacoa trees did not reduce root uptake of dimefox[59] as one would have expected if the transpiration stream carried the insecticide.

Soil application of systemics was not very efficient in the case of Systox on lemon trees[66] or of schradan on bush beans, when only 1% of the applied dose reached the above-ground parts in 5 days,[118] or with dimefox on cacao trees.[59] However, soil application at seeding time makes possible the early treatment of seedlings, and has become a popular procedure. The whole topic has been reviewed at length by Reynolds.[89]

Trunk application of systemics has been described by Jeppson *et al.*,[65, 66] using Systox on lemon trees. It was more effective than equivalent soil treatments; but 7 to 20 days were required for general translocation. Translocation after treatment in November and December was meager, but after treatment in March through October, translocation was good. This was not a purely temperature effect, for November–December temperatures were little different from those of March–April. Successful trunk application has also been shown for cacoa with dimefox,[59] Thimet, Systox, and Amiton toluenesulfonate,[13] for elm with Amiton toluenesulfonate,[107] and for coffee with dimefox.[11]

The value of systemic action in preventing rapid pest resurgence has been emphasized by Ripper *et al.*[94] They noted that schradan was selectively toxic to various pests, whereas it did not harm their predators; this then assisted both in immediate control and prevention of resurgence. Isopestox, however, failed to show this selectivity (in spite of its systemic nature) unless applied to the soil to avoid direct contact with insects. By this means only phytophagous insects were severely affected.

The translocation of Amiton and its monooxalate salt, Tetram, is of particular interest because these materials are mainly ionized at physiological pH.



$$(C_2H_{50})_2P(O)SCH_2CH_2\overset{H^+}{N}(C_2H_5)_2$$

Amiton at pH 7

Translocation in cotton and lemon plants was examined by Metcalf *et al.*[78] in 1957. Amiton and Tetram were equally well taken up from aqueous solutions applied to lemon roots (as is expected, for unless the Amiton was in sufficient quantitiy to raise the pH of the water severely, it should be present in the protonated form given above, and so should Tetram). When undiluted Amiton or a solution of Tetram was applied to the stem of the lemon or cotton, the Amiton was readily taken up, the Tetram but poorly at one-tenth of the Amiton rate; the same was true of the penetration into leaves. With trunk implantation, Bowman and Casida[13] showed that the toluenesulfonate salt of Amiton was taken up well by cacao, and Al-Azawi and Casida[1] showed that tetram was taken up well by mature elms.

These results suggest rather strongly that some liposolubility is desirable for stem and leaf penetration, but that water-soluble compounds can penetrate well through the roots or after trunk implantation. However, even this elementary simplification must be modified, for Metcalf *et al.*[78] observed that the thiono isomer of Amiton, containing the P(S)O group, penetrated cotton stems (as judged by the subsequent appearance in leaves) less effectively than the less liposoluble Amiton. Similarly, they showed in another study[76] that the thiolo isomer of Systox was six times better translocated into leaves than the more liposoluble thiono isomer, following stem application. A tentative explanation would be that for stem and leaf uptake, "intermediate liposolubility" is best: the compound should be liposoluble enough to penetrate, but water-soluble enough for subsequent translocation. Presumably for root uptake, by contrast, extreme water-solubility is desirable (if problems of loss by leaching or adsorption are set aside). These conclusions are supported by the observations of David[28] that schradan and dimefox, which are both miscible with water, were taken up well by bean roots; but schradan was taken up well from leaves, dimefox poorly, and the oil solubility of dimefox was "very much less" than that of schradan.

There has been no study devoted to the determination of what physical properties are required for systemic action. The above qualitative observations suggest the importance of solubility, but do not reveal whether the critical factors are (*a*) the absolute liposolubility for stems and leaves, and the absolute water-solubility for roots, (*b*) the ratio of liposolubility to water-solubility, or (*c*) the oil/water partition coefficient. Metcalf[71] points out that besides these penetrative requirements there would seem to be a need for sufficient water-solubility to permit movement once inside the plant, as well as (for practical purposes) an adequate stability in the plant. In Chapter 3 it was shown that most of the good *in vitro* anticholinesterases are relatively unstable unless they have a structure enabling them to bind peculiarly well to the enzyme. Spencer[104] points out that for this reason stable systemics are likely to be zoometatoxic, to use Ripper's term,[92] i.e., converted in the insect to the actual toxicant. In this way one can have a stable insecticide whose potency (and therefore instability) is only revealed when it reaches the insect. In general, then, good systemic insecticides are likely to be zoometatoxic unless they have special steric features to promote cholinesterase binding, (e.g., Tetram) or unless they have a short life in the plant (e.g., Phosdrin). A glaring exception to this rule is paraoxon, a direct toxicant with no special steric features, which is a good systemic.[29]

Metabolism

SCHRADAN

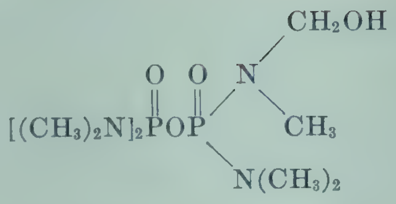
It was first shown in 1950 by DuBois *et al.*,[34] using lettuce, that plants could convert schradan to a potent anticholinesterase. A similar conversion was shown later in beans*[57] and peas.[20]

In 1954, Casida *et al.*[21] showed on the basis of partition coefficients and anticholinesterase activity that the anticholinesterase metabolite from spinach plants was identical with that from rat liver slices; in 1955, Heath *et al.*[63] used partition coefficients and hydrolyzabilities to show the identity of the metabolite produced from clover with that produced by liver slices or peroxide oxidation. From these identities and the data discussed on pages 65–67 it may be concluded that plants oxidize schradan to hydroxymethyl schradan.

* Casida *et al.*[20] believed that this finding was contradicted by other work[121] in which no increase in toxicity to mosquito larvae was found with schradan following its application to bean plants. However, there is reason to believe that the oxidized schradan is not a better larvicide than schradan,[30] furthermore, the schradan in this experiment[121] was applied to the leaves and extracted "shortly after application" so that little time for conversion was allowed.

$[(CH_3)_2N]_2P(O)OP(O)[N(CH_3)_2]_2$

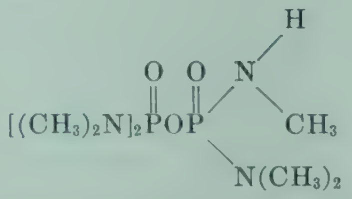
Schradan



$[(CH_3)_2N]_2P(O)OP(O)[N(CH_3)CH_2OH][N(CH_3)_2]$

Hydroxymethyl schradan

The hydroxymethyl derivative in clover was present only to a small extent (not more than 0.1 %), and another chloroform-extractable material was present in far larger amounts. This other compound was produced by several plants (clover, turnips, brussel sprouts, and French beans), was isolated in quantity from 120 kg. of turnip foliage, and was identified as heptamethylpyrophosphoramide (HMPA). It was shown for clover that at various times, from 5 to 36 days after spraying, HMPA represented a fairly constant fraction (2–7 %) of the total residue. The major residue was chloroform-inextractable material, which one month after spraying constituted 81 % of the residue.



$[(CH_3)_2N]_2P(O)OP(O)[NHCH_3][N(CH_3)_2]$

Heptamethylpyrophosphoramide (HMPA)

Heath *et al.* next discussed the route of oxidation and degradation, saying "If the degradation scheme could be represented by

Schradan → ..... → heptamethylpyrophosphoramide → ..... (1)

the maximum yield of heptamethylpyrophosphoramide could be calculated from the ratio of its half-life in the plant to that of schradan. This ratio is about 0.5, which gives a maximum of 50 %, almost independant of the orders of the successive reactions chosen for the calculation. The yields found rarely exceed 5 % at any point in the degradation. . . . Most of the degradation does not, therefore, proceed via this compound." This presumably follows from the following argument: in the hypothetical pathway shown, the rate of disappearance of schradan is given by

$$\frac{-d\,[\text{schradan}]}{dt} = k_1[\text{schradan}]$$

and the rate of disappearance of HMPA by

$$\frac{-d\,[\text{HMPA}]}{dt} = k_2[\text{HMPA}]$$

From the half-life data, $k_2 = 2\,k_1$. If the HMPA level is controlled only by the rates of schradan breakdown and HMPA breakdown, then an equilibrium will be achieved when:

$$\frac{-d\,[\text{schradan}]}{dt} = \frac{-d\,[\text{HMPA}]}{dt}$$

$$k_1[\text{schradan}] = k_2[\text{HMPA}]$$

$$\therefore\ [\text{HMPA}] = \frac{k_1}{k_2}\,[\text{schradan}] = 0.5[\text{schradan}]$$

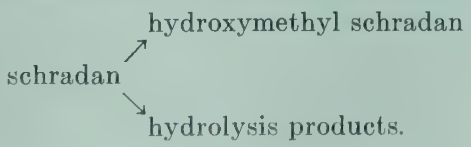
This is the maximum value that HMPA can achieve, given the above conditions. That maximum cannot be achieved if the rate of formation of HMPA is limited by the rate of synthesis or of breakdown of an intermediate between schradan or HMPA, or by the rate of synthesis of HMPA (unless the HMPA is formed directly from schradan, with no intermediate, for then

$$\frac{-d\,[\text{schradan}]}{dt} = \frac{d\,[\text{HMPA}]}{dt}$$

i.e., the HMPA level is indeed controlled by the rates of schradan breakdown and HMPA breakdown).

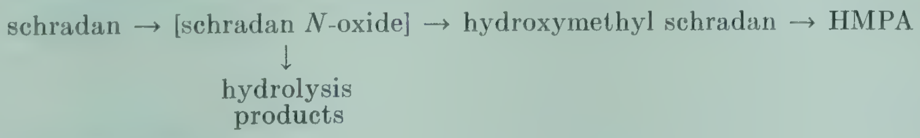
Now Heath *et al.* use the fact that HMPA was never more than 5% of the yield (i.e., 5% of the *total residue*), to refute this mechanism. But, in fact, from the very limited data, HMPA represented a much greater percentage of the *schradan level* (thus figures of 38% and 46% were found) and surely it is this percentage which matters. These figures seem to be compatible with the mechanism which Heath *et al.* reject. As discussed below, there are, however, other reasons for favoring a different mechanism.

Heath *et al.* next examined and rejected the possibility:


hydroxymethyl schradan
schradan
hydrolysis products.

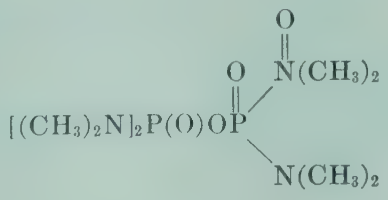
(2)

They finally proposed the pathway:

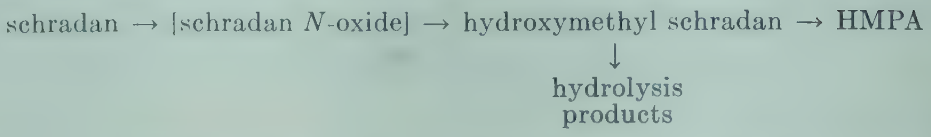

schradan
[schradan N-oxide]
hydroxymethyl schradan
HMPA
hydrolysis products

(3)

where the *N*-oxide is:


O
O
N(CH3)2
[(CH3)2N]2P(O)OP
N(CH3)2

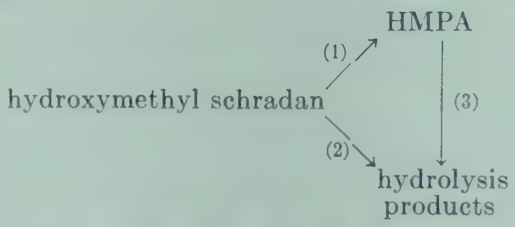
However, their arguments would equally fit the possibility:



$$\begin{tikzcd}\text{schradan} \arrow[r] & [\text{schradan } N\text{-oxide}] \arrow[r] & \text{hydroxymethyl schradan} \arrow[r] \arrow[d] & \text{HMPA} \\ & & \text{hydrolysis products} & \end{tikzcd} \qquad (4)$$

Probably in this case it would not be necessary to posit enzymic hydrolysis: Heath *et al.* report a half-life of 37 hours for hydroxymethyl schradan in water, and Spencer *et al.*[105] report a half-life of 7 hours at pH 7 and 25°C. If the *N*-oxide of schradan does exist, it would be even less stable than hydroxymethyl schradan, and therefore the route proposed by Heath *et al.* would also not require enzymic hydrolysis to account for the degradation.

The reason why one of the last two pathways (4 or 3) is preferable to the first (1) is that HMPA can only be produced via hydroxymethyl schradan, and since the hydrolysis rate of hydroxymethyl schradan is rapid, then of the reactions



reaction (2) is greatly favored; also (3) is very small compared with (2) (cf. ref.[105]). A major part of the hydrolysis must therefore precede, rather than follow, HMPA production.

In summary, it is the writer's opinion that pathway (4) above is the most probable. It is to be preferred to pathway (3) because it explains all the facts most simply, and without requiring the existence of the N-oxide as anything but a transient intermediate: this is desirable since there is no evidence for the measurable existence of the N-oxide.

Under some circumstances the available phosphorus level may influence schradan oxidation. Casida *et al.*[20] noted that with root application (but not with foliar application) increasing the available phosphorus decreased the amount of anticholinesterase produced from a given amount of schradan.

The question has been raised: is the production of hydroxymethyl schradan of importance in determining the insecticidal effectiveness of treated plants?

DuBois *et al.*,[34] in 1950, suggested that "the insecticidal action appears to be due to the active agent produced within the plant"; but there was no supporting evidence.* Casida *et al.*[21, 23] recognized that the degree of oxidation in the plant was small compared to that in the insect, and believed that the insect therefore played the major part in "toxification" of schradan, but that, nevertheless, the plant oxidation was a factor. Hartley[60] argued strongly against the probability that the plant oxidation effectively increased toxicity. It remained for DePietri-Tonelli and March[30], in 1954, to examine the matter carefully and produce experimental evidence that the small amount of active metabolite produced in plants could have "little or no importance in relation to toxicity."

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* However, O'Brien and Spencer[86] showed that insect activation could account for the toxicity of schradan.

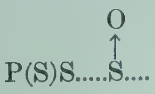
THIOETHERS

For many years the most familiar thioether systemic was Systox, with its two isomers:

$(C_2H_{50})_2P(S)OC_2H_4SC_2H_5$ Systox, thiono isomer

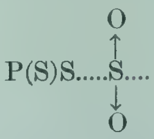
$(C_2H_{50})_2P(O)SC_2H_4SC_2H_5$ Systox, thiolo isomer

Recently, other related compounds have been used, including the P(S)S analog Disyston and its methoxy analog Ekatin, and the dithiomethylene, $P(S)SCH_2S$, analog Thimet. It will be recalled from previous chapters that this type of compound has several possible oxidation products. In the phosphorodithioates or P(S)S compounds one can have (I–V).



$P(S)S\ldots\overset{\overset{O}{\uparrow}}{S}\ldots$

Phosphorodithioate sulfoxide
(I)

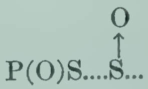


$P(S)S\ldots\underset{\underset{O}{\downarrow}}{\overset{\overset{O}{\uparrow}}{S}}\ldots$

Phosphorodithioate sulfone
(II)

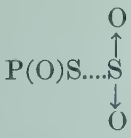
$P(O)S\ldots S\ldots$

Phosphorothiolate
(III)



$P(O)S\ldots\overset{\overset{O}{\uparrow}}{S}\ldots$

Phosphorothiolate sulfoxide
(IV)



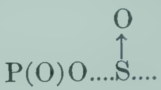
$P(O)S\ldots\underset{\underset{O}{\downarrow}}{\overset{\overset{O}{\uparrow}}{S}}$

Phosphorothiolate sulfone
(V)

In the case of Systox, one can have (IV) and (V), and also (VI–X).

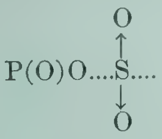
$P(O)O\ldots S\ldots$

Phosphate
(VI)



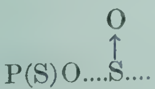
$P(O)O\ldots\overset{\overset{O}{\uparrow}}{S}\ldots$

Phosphate sulfoxide
(VII)



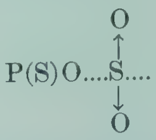
$P(O)O\ldots\underset{\underset{O}{\downarrow}}{\overset{\overset{O}{\uparrow}}{S}}\ldots$

Phosphate sulfone
(VIII)



$P(S)O\ldots\overset{\overset{O}{\uparrow}}{S}\ldots$

Phosphorothionate sulfoxide
(IX)

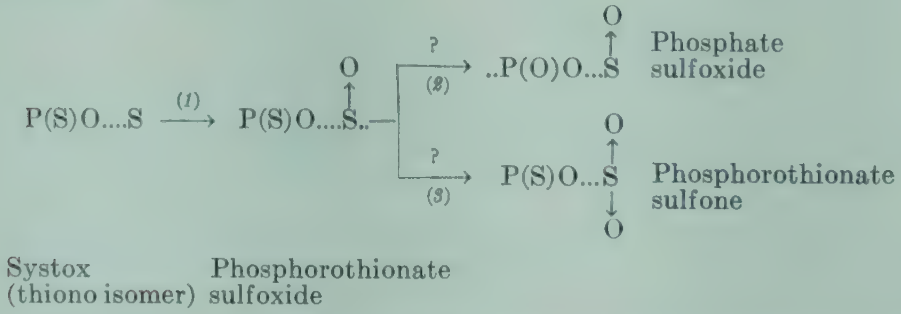


$P(S)O\ldots\underset{\underset{O}{\downarrow}}{\overset{\overset{O}{\uparrow}}{S}}\ldots$

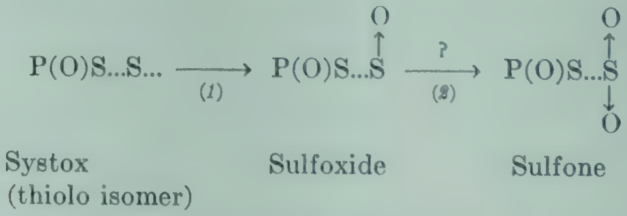
Phosphorothionate sulfone
(X)

A series of useful papers from Metcalf's group[41-43, 76, 77] has emerged over the last three years, which has clarified the plant metabolism of Systox. They first showed that when the thiono and thiolo isomers were applied separately to lemons or beans, the major product was, in each case, of a single metabolite, chloroform-extractable, and with anticholinesterase activity. The metabolite from the thiono isomer was not the same as that from the thiolo isomer. The reaction was remarkably rapid: for instance the thiolo isomer was 81 % converted to its metabolite in 24 hours using bean plants.

They next synthesized the seven possible oxidative metabolites above [(IV), (V), (VI)–(X)]. Quite good evidence was obtained (using similarities of R_f's in one paper system) that in cotton plants the thiono isomer was first oxidized to its sulfoxide. This phosphorothionate sulfoxide represented about 66 % of the dose at 1 day, but dwindled steadily to 39 % in 5 days, whereas, concurrently, another metabolite rose until it was the preponderant one (86 % in 8 days). This late metabolite could have been, according to chromatographic evidence, either the phosphate sulfoxide or the phosphorothionate sulfone.



As for the thiolo isomer of Systox, it was shown that it was oxidized either to the sulfoxide or sulfone, but these two could not be resolved.



Similar findings were made following treatment of the growing fruits of orange, walnut, and apple trees. Surprisingly enough, mere exposure of Systox isomers on glass plates to sunlight produced the same change, and with extreme rapidity; after 3 hours only traces of the parent isomers were found.

The final experiments utilized infrared spectroscopy on cotton metabolites, and clearly showed (*a*) for the thiolo isomer of Systox, the major product (98.5% at 4–6 days) was the sulfoxide, and the rest was the sulfone, and (*b*) for the thiono isomer of Systox, the phosphorothionate sulfone was a metabolite. It was impossible to say whether the phosphate sulfoxide was present, nor to evaluate what precise proportion of the metabolites which the phosphorothionate sulfone represented.

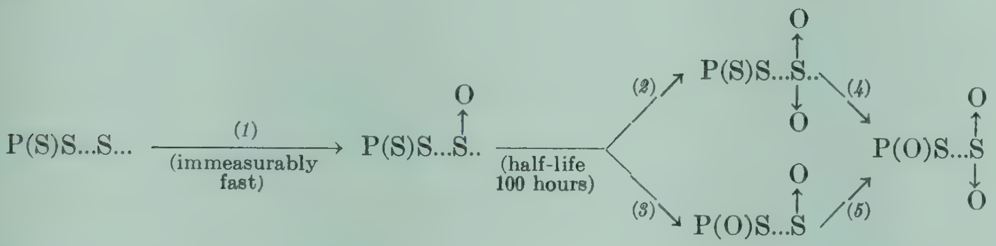
These results are in harmony with those of Heath *et al.*[63] who found in nettles and lettuce that thiolo-Systox was oxidized to its sulfoxide and later to some other product which they did not identify.

Metcalf's group[73] later turned their attention to the metabolism of Disyston (or dithio-Systox as they then called it) and Thimet.

$(C_2H_5O)_2P(S)SC_2H_4SC_2H_5$ Disyston

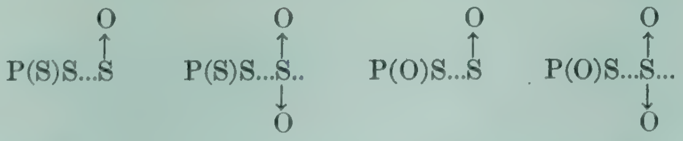
$(C_2H_5O)_2P(S)SCH_2SC_2H_5$ Thimet

When Disyston was applied to cotton plants, it was rapidly and completely metabolized in a few hours. By 75 hours, four metabolites had appeared, representing the sulfoxides and sulfones of Disyston and its phosphorothiolate oxidation product P(O)S. The sequence of reactions and some of their rates were evaluated, as follows:



Reactions (2) and (3) proceeded at approximately equal rates. Metabolism was qualitatively similar in bean, lemon, and alfalfa plants; but in alfalfa, reaction (2) was faster than (3). The results are summarized in Figs. 8.1 and 8.2.

Thimet metabolism followed a substantially similar pattern except that the parent compound persisted rather longer, and the rates of oxidation of the two sulfoxides to sulfones was measurably slower; for instance, the half-life of the phosphorodithioate sulfoxide was 200 hours as compared to 100 hours for the corresponding Disyston metabolite. Typical results are shown in Fig. 8.3 for cotton. Thimet metabolism in beans, peas, and cotton was also studied by Bowman and Casida[12, 14] using celite column chromatograms but these did not permit resolution of the following:



$$\mathrm{P(S)S...}\overset{\overset{\displaystyle O}{\uparrow}}{\mathrm{S}} \qquad \mathrm{P(S)S...}\underset{\underset{\displaystyle O}{\downarrow}}{\overset{\overset{\displaystyle O}{\uparrow}}{\mathrm{S}}}\mathrm{..} \qquad \mathrm{P(O)S...}\overset{\overset{\displaystyle O}{\uparrow}}{\mathrm{S}} \qquad \mathrm{P(O)S...}\underset{\underset{\displaystyle O}{\downarrow}}{\overset{\overset{\displaystyle O}{\uparrow}}{\mathrm{S}}}\mathrm{...}$$

However, infrared data combined with the column-position data showed

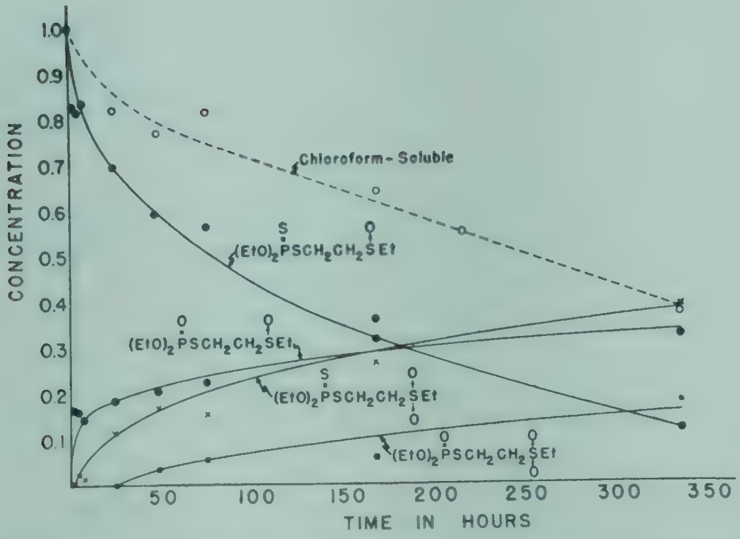


Fig. 8.1. Disyston metabolism in isolated cotton leaves. Chloroform-soluble: fraction of total radioactivity extractable into chloroform. Others: fraction of chloroform-soluble material as indicated metabolite. From Metcalf *et al.*[73]

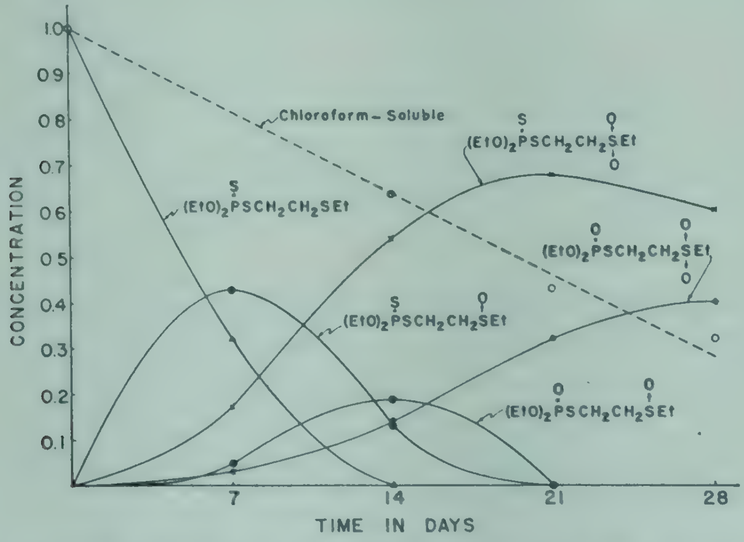


Fig. 8.2. Disyston metabolism in alfalfa plants after seed treatment. Labeling as in Fig. 8.1. From Metcalf *et al.*[73]

for cotton that there was no P(O)S...S... produced, and that all four metabolites shown in the above equation were present. There is, therefore, substantial agreement between the work of the two groups.*

* Menn[70a] found that paper chromatographic systems, including that used by Metcalf *et al.* as described above, could not resolve pure samples of the sulfoxide and sulfone of Thimet. Nor could Bowman and Casida[12] resolve them on celite columns. The anticholinesterase data of Menn and of Bowman and Casida for the sulfone are in agreement and differ from those of Metcalf *et al.* by 1000-fold. The latter authors stated that they could not purify their sulfone. Consequently it is possible that what

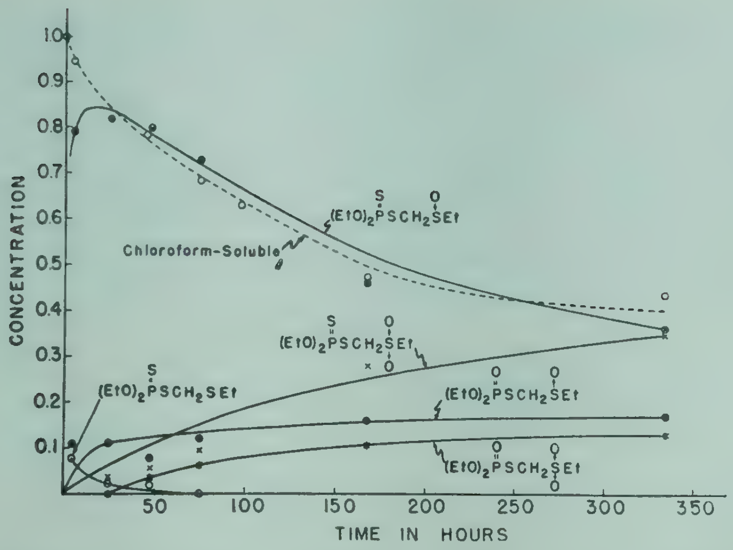


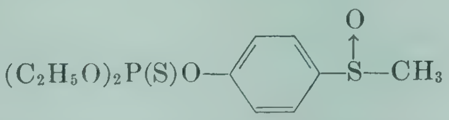
FIG. 8.3. Thimet metabolism in isolated cotton leaves. Labeling as in Fig. 8.1. From Metcalf *et al.*[73]

The data on hydrolysis of these thioethers is less comprehensive. For Disyston in cotton, the over-all hydrolysis rate conformed with first-order kinetics, the half-life being 9.4 days.[43] For Systox in citrus, bean, and cotton, hydrolysis products were derived twice as fast from the thiono isomer as from the thiolo, typical half-lives being 8 days for the thiono and 18 days for the thiolo isomer.[77]

For Thimet in beans, the hydrolytic metabolites were identified.[14] In decreasing order of initial importance they were: diethyl phosphorothioate, diethyl phosphorodithioate, diethyl phosphate, and phosphoric acid. The ratios of these products at 1 day were 8:8:2:1, at 4 days 9:2:4:3, and at 12 days 13:3:8:2 (these figures also represent the actual percentage of the total residue represented by each metabolite).

SULFINYLPHENATES

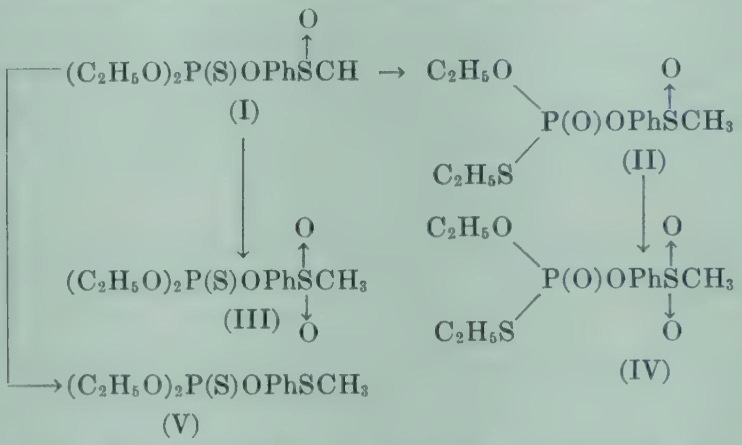
A group of compounds with certain similarities to the thioethers are the sulfinylphenates, of which the principal member is Bayer 25141:



This and similar compounds have been examined by Benjamini *et al.*[4, 5] They have systemic action, and are degraded hydrolytically: Bayer 25141 at 14 days after application is 39% hydrolyzed, and its methoxy analog at that time is 75% hydrolyzed.

Metcalf *et al.* reported in plants as the sulfone was in fact some other material, and what they reported as the sulfoxide was in fact sulfoxide plus sulfone.

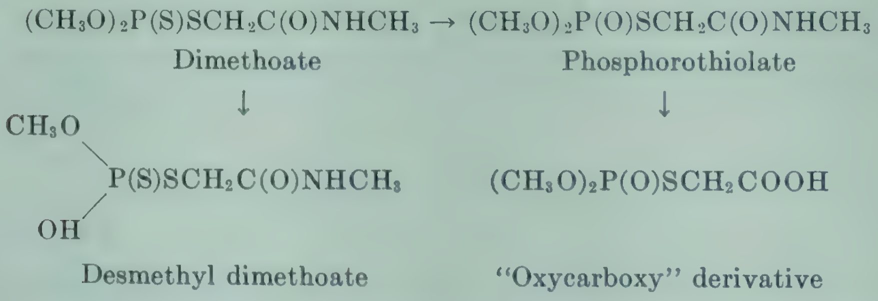
Paper chromatographic analysis of the chloroform-extractable metabolites revealed that three kinds of reaction occurred in the plant: isomerization, oxidation, and reduction. Fourteen days after applying Bayer 25141 to cotton stems, the chloroform extractable metabolites in the plant leaves consisted principally of the parent material (I) (50%) and its *S*-ethyl isomer (II) (23%), the sulfone of the parent (III) (6%) and of the isomer (IV) (6%), and there were traces of the parent's sulfide reduction product (V). Thus, where Ph stands for phenyl, the reactions were:



Surprisingly, isomerization to (II) did not occur on the plant surface, or upon glass plates, but a little of the sulfone (III) appeared. The authors suggest an enzymic isomerization, a conclusion which one cannot avoid. It represents a remarkable enzymic reaction for which there is no precedent in organophosphate biochemistry.

DIMETHOATE

The metabolism of dimethoate by corn, cotton, peas, and potatoes was examined by Dauterman *et al.*[26] following foliar application. The principal pathways of metabolism were:



There was considerable alteration of insecticide on the plant surfaces. Small but significant amounts of the phosphorothiolate and a very large amount of the "oxycarboxy" were found; for instance, on potato plants 66% of the external residue was as oxycarboxy. Inside the leaves some oxidation occurred, but in this case the major metabolite was the desmethyl deriva-

TABLE 8.1. IONIC DERIVATIVES OF DIMETHOATE IN AND ON PLANTS 12 DAYS AFTER TREATMENT[a]

Source[b]	Per cent of total ionic materials as				
	Oxycarboxy	Desmethyl	$(CH_3O)_2PSOH$	$(CH_3O)_2POOH$	H_3PO_4
Surface					
Corn	88	4	3	6	0
Cotton	71	18	5	6	0
Potato	81	9	6	4	0
Pea	9	21	11	8	52
Inside					
Corn	4	64	18	14	0
Cotton	5	69	13	13	0
Potato	10	45	18	26	0
Pea	6	18	17	12	48

[a] Data rounded off from Dauterman *et al.*[26]

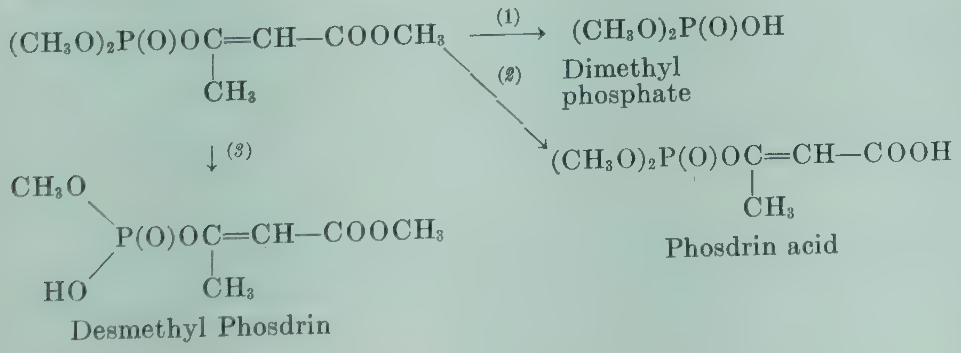
[b] Corn, cotton, and potatoes were young, 4–6 in. plants; peas were mature flowering plants.

tive, in striking contrast to the mammal, in which the carboxy derivative, $(CH_3O)_2P(S)SCH_2COOH$, was the major metabolite and the desmethyl was of little importance (page 222). The results just described were for young corn, cotton, and potato plants up to 12 days old. With peas, mature flowering plants were used, and in this case phosphoric acid was the predominant metabolite. Fuller data on the metabolites are given in Table 8.1.

PHOSDRIN

This insecticide is of particular interest as a short-term systemic poison: for example, in 13 vegetable crops the time for 90 % hydrolysis was from 0.8 to 4.2 days.[22] Thus, the possible consumer hazard is avoided, not by using a compound of low mammalian toxicity (for Phosdrin is highly toxic[44],) but by utilizing the detoxifying powers of the plant.

As Phosdrin is a phosphate, its metabolism is relatively simple, involving only hydrolysis. Three possibilities exist for the first hydrolytic step:


$(CH_3O)_2P(O)OC(CH_3)=CH-COOCH_3$
(1)
(2)
(3)
$(CH_3O)_2P(O)OH$
Dimethyl phosphate
$(CH_3O)_2P(O)OC(CH_3)=CH-COOH$
Phosdrin acid
$(CH_3O)(HO)P(O)OC(CH_3)=CH-COOCH_3$
Desmethyl Phosdrin

An additional complication is that *cis* and *trans* isomers of Phosdrin exist (page 58), and these have quite different biochemical properties. Commercial Phosdrin consists of a mixture of the isomers, whose proportions may vary from 95% *trans* to 5% *trans*, depending upon the source.[18]

The rates of decomposition of the two isomers in beans and peas were examined by Casida *et al.*[22] They found evidence for two hydrolysis product (in unstated proportions), and suggested that Phosdrin acid was the first product, which was then rapidly degraded to dimethyl phosphate. No direct evidence for this route was presented.

Spencer and Robinson[106] used paper chromatography to show that when *cis*- or *trans*-Phosdrin were applied to pea plants by the roots, the principal metabolite at 48 hours was dimethyl phosphate (about 99%), and the remainder was Phosdrin acid. Desmethyl Phosdrin and desmethyl Phosdrin acid were absent, although trace amounts would have escaped detection. They next applied *cis*Phosdrin acid to plants, and found that it was a little more stable than *cis*Phosdrin, and it was degraded to dimethyl phosphate and desmethyl Phosdrin acid in the ratio of 4 to 1. These data could be used qualitatively to support either of two possible pathways:

(*a*).

$$\begin{tikzcd}
\text{Phosdrin} \arrow[r] & \text{Phosdrin acid} \arrow[r] \arrow[dr] & \text{dimethyl phosphate} \\
 & & \text{desmethyl Phosdrin acid}
\end{tikzcd}$$

(*b*).

$$\begin{tikzcd}
\text{Phosdrin} \arrow[r, "k_1"] \arrow[dr, "k_2"'] & \text{dimethyl phosphate} & \\
 & \text{Phosdrin acid} \arrow[r] & \text{desmethyl Phosdrin acid}
\end{tikzcd}$$

However, if pathway (*a*) were followed, then every four moles of dimethyl phosphate produced by Phosdrin degradation should be accompanied by one mole of desmethyl Phosdrin acid. In fact, none but trace amounts of desmethyl Phosdrin acid could have been produced. Such a finding makes (*a*) impossible; instead (*b*) must be the route, and k_1 must be much larger than k_2.

Residues

The data upon plant residues of organophosphate are quite extensive, but not well-suited to discursive treatment. The conditions of application (dose and method), plant type, and weather are so diverse as to turn a discussion into a catalogue. Therefore, instead of discussing the topic, Table 8.2 is herewith offered giving a few references for some of the major insecticides. Relevant reviews include those of Gunther and Blinn[50] and Reynolds.[88]

For the principal methods used for residue analysis, the comprehensive text of Gunther and Blinn[49] may be recommended. More recent chemical

TABLE 8.2. RESIDUE DATA FOR PLANTS

Insecticide	Plant	Analysis[a]	Ref.
Delnav	Cabbage, bean	*f*	19
	Lemon, orange	*t*	53
Diazinon	Cherry	*t*	87
	Orange, lemon	*t*	52
Dimefox	Hops	*t*	68
	Walnuts	*t*	10
Dimethoate	Corn, cotton, pea, potato	*d*	26
	Beans, cheries	*d*	96
Disyston	Alfalfa	*d*	73
	Cotton	*d*	90
EPN	Apple	*t*	38
	Peach	*t*	38, 15
Malathion	Apple	*t*	119
	Alfalfa	*t*	32
	Asparagus	*t*	31
	Date	*t*	69
	Lettuce, tomato, onion	*t*	103
	Orange	*t*	9
	Spinach	*t*	55
	Turnip, collard	*t*	115
	Vegetables, berries, tobacco	*t*	116
	Wheat	*t*	54
Methyl parathion	Apple	*t*	38
Parathion	Alfalfa	*t*	64
	Apple	*t*	38
	Celery	*t*	81
	Citrus	*t*	17
	Cucumber, tomato	*t*	10
	Date	*t*	69
	Lettuce	*t*	99
	Peach	*t*	15, 38
	Spinach	*t*	55
	Strawberry	*t*	33
	Tomato	*t*	101
Phosdrin	Cotton	*t*	22
	13 Vegetables	*f*	22

TABLE 8.2.—*Continued*

Insecticide	Plant	Analysis[a]	Ref.
Schradan	Clover, turnip	*f*	63
	Cotton	*f*	74
	Hops, sprouts, strawberries, sugar beets	*f*	62
	Mustard, borage, and their nectar	*t*	48
	Orange, lemon	*t*	75
Sulfotepp	Lettuce, tomato	*t*	101
Systox	Beans	*f*	109
	Collard, lettuce, mustard	*t*	79
	Lemon	*t*	76
	Lima beans	*t*	120
	Orange, apple, pear, walnut, potato	*t*	77
	Pea	*t*	2
	Peaches	*t*	37
	5 Vegetables	*t*	80
	Vegetables, fruit, forage	*t*	16
Tetram	Cacao	*t*	13
	Orange, lemon	*t*	78
Thimet	Alfalfa	*d*	90
	Cotton	*d*	73
	Cotton	*f*	12
	6 Vegetables (averages)	*t*	12
Trithion	Orange, lemon	*t*	51

[a] *t* = total residues; *f* = fractionation of residues; *d* = detailed identificati

methods have been reviewed by Schechter and Hornstein,[97] and bioass methods by Sun[108] and Nagasawa.[54] Since these reviews, methods have be developed for Chlorthion,[67] dimefox,[59] Disyston,[53] Guthion,[46] malathion parathion,[36, 40] and Thimet.[82]

Phytotoxicity

Because insecticides are commonly applied to plants for their prote tion, it is of considerable importance that phytotoxicity should be negli ble. There is, however, relatively little published data on this importa topic, and the degree of interest (as judged by publications) seems l now than it was eight years ago. This may be due, in part, to the increas work done by manufacturers before releasing a compound, so that much the phytotoxicity data is given briefly in bulletins accompanying the offic birth of the compound.

One might take the view that knowledge of which plants are affected by and which are unharmed by the various insecticides is sufficient. Indeed, this is about all the information the following pages can supply. In the writer's opinion such limited knowledge is quite insufficient. Nobody has yet examined the properties of organophosphates which bestow phytotoxicity, nor made any attempt to account for the clear-cut differences in susceptibility between various plant species. This work is now long overdue.

We now proceed to the task of cataloguing a few of the observations which have been made upon the phytotoxicity of some organophosphates.

DIMEFOX

Dimefox was harmless to turnips when applied as a 0.2 % spray; when the turnips were grown in dimefox solutions they were moderately damaged at 0.05 % but not at 0.01 %.[29] For cacao seedlings[59] 0.02 gm. per plant (as a 20 % spray) caused no ill effects, but at 0.1 gm. per plant leaf fall was found, and 0.6 gm. or over gave total defoliation. The plants were not killed, however, and later leaves developed normally. (For persistent control of mealy bugs, 0.4–0.7 gm. per tree were needed.) Mature coffee trees were completely unaffected by trunk and root application of dimefox, applied up to 6 gm. per tree to the trunk or 12 gm. per tree to the soil.[11]

GUTHION

Guthion on insect-free cotton at 0.25 lb. per acre had no injurious effect, and actually increased flower formation somewhat, from the usual 16 to 19.3 flowers per plant. Higher levels (0.5 and 1 lb. per acre) were without any effect.[56]

MALATHION

The susceptibility of 14 tree species to malathion spray (3 %) has been reported;[24] damage was noted in every case, was severe in seven cases, and was lethal in one species. In another series, using 5 % sprays, there was a remarkable variation in sensitivity: for example, silver maple and white pine trees were affected very little, hickory and wayfaring trees were killed.

Malathion "shows little phytotoxicity to most plants raised in the greenhouse," except for poinsettias.[61] At concentrations needed to control mealy bugs on these plants (0.1 to 0.2 % spray), curling, yellowing, and leaf drop were observed.

PARATHION

An extensive study was made by Scott[98] upon the phytotoxicity of parathion to over 37 ornamental tree species. A 0.02 % spray gave no damage with fully growing plants, but if applied to dormant plants (deciduous or evergreen) having tight terminal buds, gave rise to light to severe damage. McIlrath[70] noted that parathion at "three times the recommended insecticidal dosage" caused an increase in the number of leaf lobes. At higher levels, parathion caused "burning" but no increase in lobe number.

In 50 greenhouse plants, no phytotoxicity of parathion was found, even in those species susceptible to TEPP.[102] Burning of certain cucumber varieties but not others has been reported for 1 % parathion, and only slight burning of one variety with 0.5 %.[25]

Parathion's phytotoxicity for apples was examined by Glass[47] with both greenhouse and field material. With normal insecticidal levels (e.g., 1 lb. per 100 gal.) 6 varieties of 22 studied were seriously injured. The injury was worst with young leaves, which showed severe necrosis; leaf drop followed. Fruits were severely spotted. The other 16 varieties were not affected by less than 5 lb. per 100 gal., and in some cases not by 20 lb. per 100 gal., and these resistant varieties showed rolling or cupping of thier leaves rather than necrosis. Injury in all cases was prevented by formulating the parathion with activated bentonite or activated charcoal; this prevented the formation of droplets of free parathion which otherwise existed in suspensions of parathion wettable powder.

Tomatoes (3 varieties) were not affected by normal doses of parathion (0.5 lb. per 100 gal.). Larger amounts (e.g., 2 lb. per 100 gal.) caused two kinds of injury: a prompt marginal bleaching and a delayed necrotic spotting. The bleaching was caused by impurities in the technical parathion, the spotting by parathion itself.[35]

The succulent ornamental *Crassula multicava* was very susceptible to parathion. Even at 0.5 lb. per 100 gal. there appeared, within 5 days, watery patches on the lower leaf surfaces and necrosis at the bases of the growing tips, and death occurred within 10–14 days.[35]

Tobacco seedlings grown in soil previously treated at 0.24 lb. per acre of parathion were unharmed, nor was direct treatment of the seeds with dilute suspension or solutions of parathion harmful; but three-week seedlings treated at the enormous rate of 23 lb. per acre were severely scorched and many were killed.[3]

SCHRADAN

There is considerable variation in the susceptibility of plants to schradan. Hops, sugar beets, brussel sprouts, marigolds, and cabbages are not usually damaged (in the case of sprouts a 20 % spray caused no damage). Peas, strawberries, chrysanthemums, roses, blackcurrants, and clover show intermediate susceptibility, being damaged by sprays over 1 %, but not by 0.5 %. Potatoes and beans show "scorching" with schradan, and apples may suffer from defoliation and fruit drop—but not from scorching.[93, 95] Lemon plants are extremely resistant: plants grown in water culture were induced to take up schradan to give 6 mg. of schradan per gram of leaf, without any trace of injury.[75] The same was true of turnips grown in a 0.5% solution or sprayed with a 0.2% solution.[29]

The effects of soaking seeds in schradan have been reported by Tsi.[113] Some small stimulation of subsequent growth was found at low levels of schradan; but with 2% solutions or stronger, inhibition was found with cotton, peas, and beans. Nasturtiums, however, tolerated 5% solutions without effect.

A study by Casida *et al.*[20] of the effect of schradan upon pea plants showed a relationship between inhibition of growth (measured as total weight gain) and concentration of schradan in the nutrient solution for the concentration range 0.03–10.0% (it was stated however, that in some experiments a stimulation of dry weight gain was observed with low concentrations). The phytotoxic symptoms at high levels were chlorotic spots and marginal necrosis, which were seen first on the lower leaves, and only in extreme cases in the terminal bud region. Available inorganic phosphorus levels did not influence phytotoxicity. The phytotoxic effects were more severe with technical than with equivalent pure schradan.

The biochemical consequences of schradan phytotoxicity have been briefly examined.[20] Respiration of bud tissue was inhibited a little by low concentrations of schradan in a nutrient medium, (e.g., 7% inhibition by 40 p.p.m.) but not by high concentrations (1000 p.p.m.). Phosphatase activity was inhibited somewhat, to an extent increasing with increasing schradan concentration: 1000 p.p.m. caused 25% inhibition. Extracellular root phosphatase was also inhibited: at 23 p.p.m. it was inhibited 6% in 0.5 hours and 37% in 16 hours. This inhibition could be reversed by washing.

TEPP

Hall[58] observed that TEPP applied early in flower development caused carnations to branch repeatedly and produce small inferior flowers; when applied later it caused hypertrophy of stamens and pistils and inhibited petal development. These effects were obtained with high concentrations of TEPP: 600 p.p.m. sprayed to run-off. Applied to tomatoes, levels above 2000 p.p.m. were lethal: wilting was observed within 2 hours, the foliage dried out, and the plants were dead in 1 day. At levels of 800–2000 p.p.m., the plants were either killed or their apical meristem was affected, so that lateral branches developed. At lower levels (e.g., 100–400 p.p.m.) a prompt stimulation of respiration and of development occurred, so that the time to flowering was reduced. Hall emphasized the similarity of these effects to those caused by the growth-regulating substance, 2,4-dichlorophenoxyacetic acid.

TEPP was shown to cause malformation of cotton leaves when used at

15–30 times the concentration required for insect control: the malformation at its most extreme involved very severe shrinkage and wrinkling. Preparations of "HETP" (a TEPP-containing mixture) caused worse damage than their equivalent of pure TEPP. Plants grown during the winter were particularly susceptible.[70] Using normal insecticidal levels of HETP,[102] only tomatoes and chrysanthemums were affected, of 180 species examined; of the chrysanthemums only 30 out of 140 varieties were sensitive. If the tomato or chrysanthemum plants were young or succulent, they were particularly susceptible. Tomato injury was as small water-soaked spots, which later necrosed. Chrysanthemum injury was as scattered black dots, which later showed a pale halo; eventually the whole leaf yellowed.

In roses, the stimulation of growth caused by HETP was so great as to suggest a direct stimulation apart from that caused by spider mite control.[100]

Of 18 flower and fruit species sprayed with the enormous concentration of 30% TEPP, only tomato and *Kalanchoë daigremontiana* were killed, although 13 other species were damaged or defoliated.[122]

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CHAPTER 9

Selective Toxicity

In all the peaceful uses of organophosphates, selective toxicity is desirable. The simplest requirement is that all the pesticides be nonhazardous in use; in this case, the desired selectivity is usually against arthropods as opposed to mammals. The next requirement arises out of the observation that the use of nonspecific pesticides commonly leads to ultimate pest levels in excess of the original outbreak, as a result of the unavoidable destruction of the pest's predators and parasites.[40, 41] Consequently, the professional entomologist would often like a pesticide specific to one arthropod species. This desire must, in practice, be strongly modified by economic considerations, for manufacturers have little interest in producing pesticides of very restricted use.

These entomologic and economic factors must be taken into account in the practical application of the selectivity theory. In this chapter we shall, however, neglect these factors and discuss only those factors that contribute to selectivity of any kind. In particular, an attempt will be made to show how the great mass of information given in the preceding eight chapters can be utilized to design organophosphates having predictable selectivity. Such an aim represents, in the writer's opinion, the ultimate level of the understanding of the organophosphates. When we know 90% of the important facts about how organophosphates act, we may be 90% successful in designing new compounds. At present, we are nowhere near that goal, and must expect our designing abilities to be correspondingly inadequate. One of the best ways of advancing our understanding may be the attempted preparation of compounds with predictable properties: most profit will emerge when the attempts are unsuccessful, for in seeking the reasons for this lack of success, a deeper understanding will be achieved.

A special case of selectivity is the phenomenon of resistance, in which a toxicity difference comes to exist between two strains of a single species. This is one of the rare cases where selectivity is undesirable. The problem can be satisfactorily tackled only when the reason for the selectivity is understood.

There are many ways by which selectivity may be achieved. Ripper *et al.*[42] call selectivity "ecological" when it is caused by differences of behavior or habitat: in this way phytophagous insects would be killed by a systemically poisoned plant, other insects would escape. Localized application of spray and the use of protective clothing by operators are both devices by which "ecological selectivity" is obtained. The other source of selectivity was termed "physiological" by these authors, and applies to every case of selectivity which is shown when poison is applied directly and equally to susceptible and nonsusceptible species. In such a case, selectivity can be caused only by a difference in the penetration of the poison into the animal, or in subsequent events. Thus, physiological selectivity can be due either to absorptive effects or to what we may call "intrinsic selectivity," i.e., a selectivity that operates even when the poison is delivered inside the animal body.

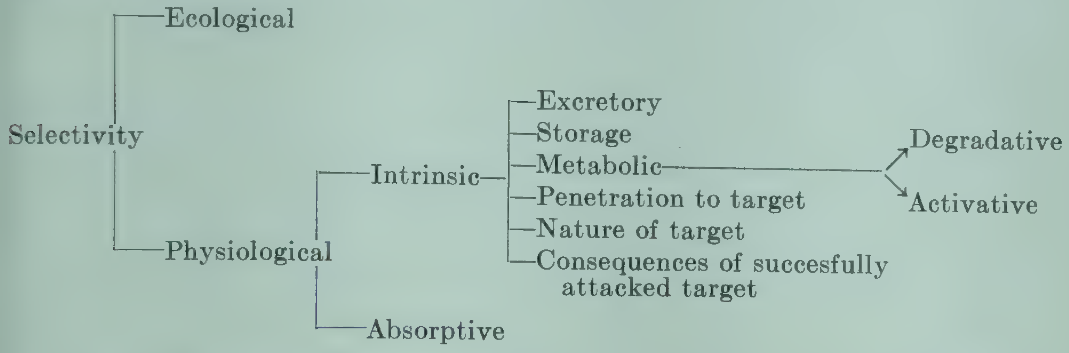
When one compares toxicity data obtained by topical or cutaneous application with different species, the differences are found to be due to physiological selectivity. If instead, one compares data obtained by injection, for example, into the hemocele of insects and the peritoneum of mammals, any differences are found to be due to intrinsic selectivity. Table 9.1 gives some mixed data, partly of unspecified physiological selectivity (comparing injected mice with topically treated insects), and partly of intrinsic selectivity (all animals injected). From the table one can see various forms of strong selectivity. It is our business to account for these.

TABLE 9.1. SELECTIVE TOXICITY OF SOME ORGANOPHOSPHATES[a]

Compound	Mammal used	Housefly treatment: injected (*i*) or topical (*t*)	LD_{50} mammal/ LD_{50} housefly
Selective insecticides			
Malathion	Mouse	*t*	68
Dimethoate	Mouse	*t*	325
Co-ral	Mouse	*t*	16
Diazinon	Mouse	*t*	27
Dipterex	Rat	*t*	31
Selective mammalicides			
Schradan	Rat	*t*	0.009
Iso-Systox methosulfate	Mouse	*i*	0.03
Tetram	Mouse	*i*	0.00042
Ro 30412	Mouse	*i*	0.004
Nonselective compounds			
Phosdrin isomers	Rat	*t*	0.8
Parathion	Mouse	*t*	6
DFP	Rat	*t*	9
Thimet	Rat	*t*	6
TEPP	Mouse	*t*	0.17

[a] LD_{50}'s at 24 hours. Mouse: intraperitoneal application. Rat: subcutaneous application. Data from references 6, 18, 21, 23, 25, 30, 34, 39.

The causes of intrinsic selectivity can be subdivided as in the accompanying diagram.



This is intended to be a complete list of the factors which, if different in two animals, can cause selectivity. If the list is complete, then selectivity must always be due to a difference in one or more of these factors. There is, therefore, a logical sequence of approaches possible in studying selectivity, since all but two of the factors in the diagram can be readily studied by techniques now available. The two factors about which an answer cannot be guaranteed are: the nature of the target (by which is meant the critical target, not merely any system which is attacked), and the consequences of the inhibition of the target. Our knowledge about this latter factor is rather extensive for the acutely poisoned mammal (page 176) but nonexistent for the insect. We simply do not know what is the ultimate cause of the insect's death. Consequently, this factor will not be discussed below. The other factors will be considered in order.

Absorption

The integuments of arthropods differ so widely from one another, and are so unlike the mammalian skin, that one would suppose selectivity caused by absorption differences would be a commonplace occurrence. However, it seems that with lipophilic organophosphates, the integument or skin provides very little opposition to entry. The opposition can be roughly measured by what we may call the permeability factor *P*, defined for insects as LD_{50} (topical)/LD_{50} (injection.) This should have a minimum value of 1 if permeability is rapid. For the American cockroach, the *P* for parathion is 1.2, for paraoxon 1.1, for Diazinon 2.7, for dimethoate 2.0, and acethion 1.9.[21, 25] Unfortunately, *P* does not indicate permeability alone, but rather an interaction between penetration, detoxification, and the importance of rapid attack. For instance, if a phosphate is not detoxified, and if it does not matter whether it is delivered slowly or rapidly to the target, *P* would be 1 even if the penetration rate was extremely slow. If the rate of delivery to the target is unimportant, (as may well be true within a few hours for diethyl or diisopropyl phosphates, where reactivation is not important) but detoxification is brisk, *P* becomes a good measure of permeability: when permeability is very slow, *P* will be very high, as the phosphate may be detoxified as rapidly as it penetrates.

In one case, selectivity has been clearly attributed to absorption. Malathion is 8 times as toxic to the American as to the German cockroach by topical application, but only 1.1 times as toxic by injection. Furthermore, injected malathion was metabolized in the same way by both insects. Only absorption differences could account for such results.

The same arguments apply to the mammal. The data here are even sketchier than for the insect. If this time, for the lack of injection data, *P* is defined as the ratio of LD_{50} (cutaneous)/LD_{50} (oral), two different orders of magnitude for *P* can be found. For relatively lipophilic compounds, values in the rat were 1.6 for parathion and 2.3 for Systox. For relatively hydrophilic compounds, values in the rabbit were 31 for schradan and 17 for DFP.[25] Presumably, then, the skin is only a good barrier to hydrophilic compounds.

The importance of the rate of skin penetration, i.e., of delivery into the body, is suggested by a study done by Saunders[43] who found that the rate of intravenous injection of DFP and TEPP was linearly related to the LD_{50}.

Selectivity by absorption differences offers little hope for the development of low-hazard insecticides, for oral intake as well as skin contact must be made innocuous. However, if one could build into an organophosphate the capacity to be degraded in the mammalian stomach, for instance, by introducing an acid-hydrolyzable group, and also make it considerably hydrophilic without being actually ionic,* one might achieve a compound of low oral and cutaneous toxicity. It would, presumably, have little contact insecticidal action because of its low cuticular penetration; and would be quite systemic: these factors would confer some "pest selectivity." This design utilizes the fact that the insect gut is never very acid, in fact it is usually neutral or alkaline.[49]

One might hope that absorption differences could be utilized to obtain selectivity between different arthropods, in view of the selection between the American and German roach reported above. A thorough investigation of the variations in permeability among arthropod species is needed. In such a study it will be important to bear in mind the fact that the measurement of the rate of disappearance of a topically applied insecticide from the insect's surface does not necessarily measure the rate at which the material effectively enters the body. For instance, malathion, according to indirect evidence, must enter the body more rapidly in the American than

* Ionic organophosphates are liable to be poor insecticides, see page 334.

in the German roach, but its rate of disappearance from the surface, as judged by acetone rinsing, was the same in both species.[20] Presumably, material can be held somewhere in the integument, unavailable either to acetone washing or to the hemolymph. In order to examine this effect experimentally, one would have to devise a fractionation technique to separate the integument from the other tissues.

A special form of absorptive selectivity involving differences in absorption (or uptake) from the vapor phase is described on page 264 for the case of insect eggs. There appears to be an important difference in the amount of parathion taken up by the eggs of the milkweed bug and the eggs of the peachtree borer, and this difference reflects a difference in susceptibility. Now, size of egg alone should influence surface uptake, for the surface area in centimeters per gram of a large egg is less than that of a small one. Consider two spherical eggs, identical in the absorptive nature of their surfaces, but differing fivefold in weight. Assuming that their average densities are the same, their surface areas are directly proportional to their weights (since both are proportional to the radius squared, r^2). The small egg will, therefore, absorb 5 times as much (in micrograms per gram) as the large egg. It is likely that the nature of their surface lipids will also have a profound influence upon uptake, and that the degree of selectivity will also depend upon the physical properties of the toxicant.

To counteract the unpleasantly hypothetical aspect of the above paragraph, it is a welcome observation that in the case of parathion at least, the majority of the compound, following fumigation of the eggs, is found in the lipoidal surface of the chorion,[35] so that it seems probable that uptake into this surface layer is of prime importance in determining toxicity. Consequently, one may hope that in this case selectivity can be reliably correlated with the geometry and physical properties of a clearly defined component of the organism.

Next we will consider, in order, the forms of what is called in the classification on page 317 intrinsic selectivity, that is, selectivity which operates even when the toxicant is administered by injection in order to eliminate differences in penetration through skin or integument.

Excretion and Storage

These two aspects have only one thing in common: they have no known importance as factors in selectivity.

The mammal has a urinary system capable of disposing of ionic materials with remarkable rapidity. However, as Brodie *et al.* point out,[7] urinary excretion is of little importance in disposing of lipid-soluble compounds, into which class fall all the important organophosphates. And indeed as Chapter 6 has shown, unaltered organophosphates are not found in the urine of treated animals. Since the excretable products of organophosphate metabolism are nontoxic, excretion cannot play an important role in determining the toxicity of these compounds.

The few ionic organophosphates which are toxic (e.g., Tetram, phosphopyristigmine) usually have selectively mammalicidal action, and excretion cannot, therefore, provide protection —although it is always possible that such compounds would be even more mammalicidal were urinary excretion to fail.

Storage should influence toxicity: it seems likely that extensive fat depots in an animal should withhold organophosphates from distribution to more vital places, and reduce their blood level. There is no record of research on this topic, and it is unlikely that storage differences can contribute much to selectivity between species.

Metabolism

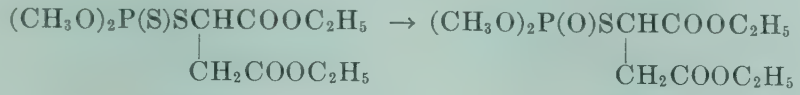
Our understanding of selectivity is most advanced in the cases of metabolic selectivity, and it is in this area that the best chances exist for designing new compounds with predictable toxicity patterns.

CARBOXYESTERS

Malathion has been the compound most studied in attempts to explain selectivity. It has the useful properties of good insecticidal activity with such a low mammalian toxicity that accidental poisoning is almost impossible. By intraperitoneal injection into the mouse the LD_{50} is 815 μg./gm., and by topical application to houseflies it is 12 μg./gm., a selectivity of sixty-eightfold.[38]

In 1956, March *et al.*[22] proposed that "The less effective metabolism in the insect may explain the great differential in toxicity for malathion between insects and warm-blooded animals." This conclusion has since been amply confirmed, but the data on which it was based at the time now appear somewhat insufficient: in the paper chromatogram of an extract of the gut of a cockroach poisoned with 1000 μg./gm. of radioactive malathion, March *et al.* found only one metabolite after 2 hours, in contrast to six metabolites found in chicken feces or mouse urine. The actual rate of degradation in the insect was not studied. Later studies with the American cockroach[20] have shown that, in fact, eleven metabolites are produced in the whole insect, as compared to seven in the whole mouse.

A different approach was used in 1957 by O'Brien[28] who added malathion to liver slices, which are known to be very effective in oxidizing phosphorothionates to phosphates. One might have expected the following reaction:

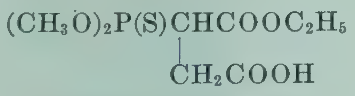


$$(\mathrm{CH_3O})_2\mathrm{P(S)S}\underset{\mathrm{CH_2COOC_2H_5}}{\underset{|}{\mathrm{C}}}\mathrm{HCOOC_2H_5} \rightarrow (\mathrm{CH_3O})_2\mathrm{P(O)S}\underset{\mathrm{CH_2COOC_2H_5}}{\underset{|}{\mathrm{C}}}\mathrm{HCOOC_2H_5}$$

Malathion Malaoxon

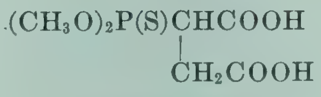
In fact, no accumulation of malaoxon could be detected (using an anticholinesterase assay). Furthermore, added malaoxon was rapidly degraded by these slices. It seemed then that malaoxon in liver would be destroyed as fast as it was produced. In cockroach preparations, by contrast, malathion was slowly converted to malaoxon, and as a corollary, added malaoxon was only slowly degraded. It was concluded that "the nontoxicity of malathion to the mouse is due to the vigorous hydrolytic degradation of either malathion or malaoxon at the carboxylic ester link." This conclusion was somewhat speculative, as it was based on *in vitro* findings. The view that degradation was at the carboxylic link was pure guesswork, the guess being based on the two special features of malathion: its low toxicity and its carboxyester group.

The final confirmation of the mechanism of malathion's selectivity came in 1959, when Krueger and O'Brien[20] applied radioactive malathion to various insects and the mouse, identified the degradation products, and followed the time-course of malathion disappearance and malaoxon appearance.* Some of the data on the nature of the hydrolysis products are given in Table 9.2, which shows that in insects the products are primarily those of phosphatase action; in the mouse they are primarily those of carboxyesterase action, leading to malathion monoacid and (under other conditions than those of Table 9.2) diacid.



$$(\mathrm{CH_3O})_2\mathrm{P(S)}\underset{\mathrm{CH_2COOH}}{\underset{|}{\mathrm{C}}}\mathrm{HCOOC_2H_5}$$

Malathion monoacid



$$(\mathrm{CH_3O})_2\mathrm{P(S)}\underset{\mathrm{CH_2COOH}}{\underset{|}{\mathrm{C}}}\mathrm{HCOOH}$$

Malathion diacid

Extensive studies carried out on the urine of the dog, the rat,[16] and the cow[33] after malathion treatment gave results for the proportion of various metabolites similar to those for the whole mouse in Table 9.2. Thus, the carboxyesterase products in urine at various times after treatment were 53–67 % of the total for the dog, 59 % for the rat, and 69–80 % for the cow.

The differences between the percentages as phosphatase products and carboxyesterase products are not in themselves sufficient to account for the difference in toxicity for, say, the American cockroach and the mouse.

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* It is the writer's view that such a study, elaborate though it is, is obligatory if selectivity is to be attributed reliably to a metabolic difference. Techniques for these *in vivo* experiments are now available (Chapter 10).

TABLE 9.2. NATURE OF MALATHION HYDROLYSIS PRODUCTS IN INSECTS AND THE MOUSE[a, b]

Compound	American cockroach		German cockroach		Housefly		Mouse	
H_3PO_4	5	29	0	44	0	49	0	21
$(CH_3)_2POOH$	6		8		7		3	
$(CH_3)_2PSOH$	12		22		36		13	
$(CH_3)_2PSSH$	6		14		6		5	
Malathion monoacid	42	50	43	43	27	27	68	68
Malathion diacid	8		0		0		0	
Unknowns	21		13		24		12	

[a] Figures are as % of the total hydrolysis products found 0.5 hour after doses (related to the LD_{50}) in μg./gm. to American roach 4, German roach 60, housefly 15, and mouse 30. The results are altered very little by tenfold increases in dose. Insects: topical application. Mouse: intraperitoneal application.

[b] Data of Krueger and O'Brien.[30]

One should be able to show that total degradation was more extensive in the mammal, and that as a consequence the level of the actual toxicant, malaoxon, was reduced. Figure 9.1*a* shows the variation with time of chloroform-extractable materials, i.e., total unhydrolyzed materials, mainly malathion, in whole animals. There is a difference, but a relatively small one, between the cockroach and mouse. However, there is an extremely large difference between the levels of malaoxon in these species, and such a difference in levels of actual toxicant seem amply sufficient to account for the differential toxicity.

From this malathion study, it can be concluded that the differential toxicity of malathion is due to a different balance of activating and degrading enzymes in the susceptible and nonsusceptible species, which leads to much greater levels of malaoxon in susceptible animals. Probably the most important factor is more extensive carboxyesterase activity in the mammal (nonsusceptible).

An indirect confirmation of this view comes from the success of a deduction based upon it. It was argued that the carboxyesterase(s) that was responsible for the extensive degradation in the mammal could not be very specific, for malathion is an exotic compound. Other organophosphates containing a carboxyester should also be degraded faster in the mammal than in the insect. Such a degradation, if arranged so that it left a carboxylate ion near the phosphorus, should constitute a detoxification, because of the large nucleophilic effect of an anion upon the phosphorus (page 93). Another factor must be considered: if an organophosphate acts rapidly, detoxifying systems may be of little importance: thus, DFP is very toxic to mammals in spite of high DFP-ase levels in various tissues (page 117). With phosphorothionates, as opposed to phosphates, there is a lag period whereas the actual toxicant, the phosphate, is being produced by P═S oxidation. This lag period gives an opportunity for detoxifying systems to operate. This factor has, for this reason, been called "the opportunity factor."[31] From these arguments, a hypothesis emerged in 1957: the introduction of a carboxyester group near the phosphorus atom of a phosphorothionate whose phosphate is a good anticholinesterase should bestow the property of selective toxicity to the insect as compared with the mammal.[27]

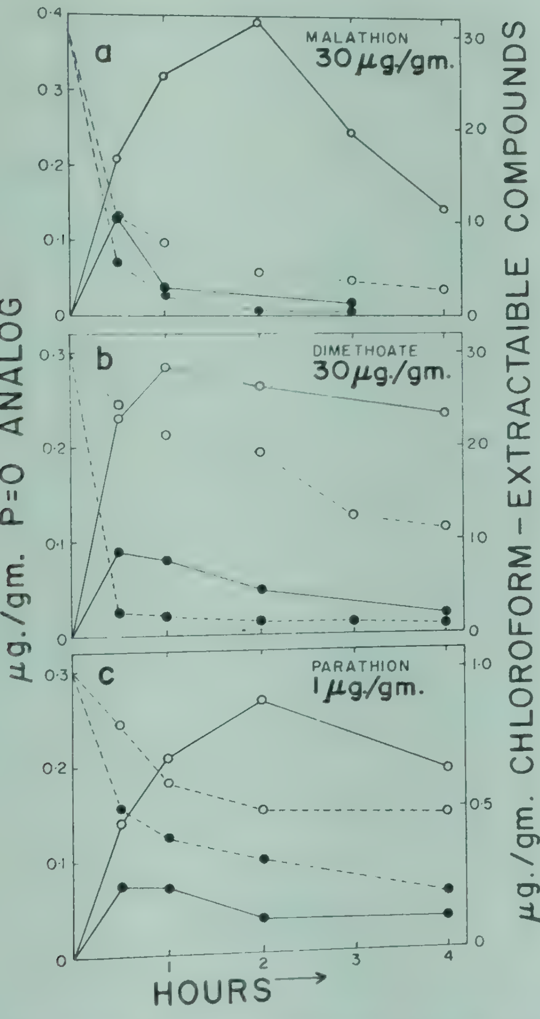
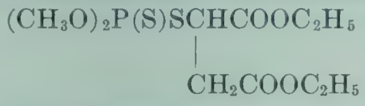


FIG. 9.1. (*a*, *b*, *c*). Levels of chloroform extractable compounds (– – –) and phosphate analog (——) in the American cockroach (○) and the mouse (●) after poisoning. The chloroform extractable compounds consist mainly of the parent compound, whose precise level may be obtained by subtracting the phosphate analog level from the chloroform-extractable level. From Krueger *et al.*[20, 21]

TABLE 9.3. SELECTIVE TOXICITY OF NEW ORGANOPHOSPHATES CONTAINING CARBOXYESTER GROUPS[a]

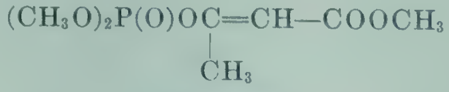
Compound	Structure	L_1 = LD_{50} mouse	L_2 = LD_{50} housefly	L_1/L_2
Acethion	$(C_2H_5O)_2P(S)SCH_2COOC_2H_5$	1280	9.4	136
Acetoxon	$(C_2H_5O)_2P(O)SCH_2COOC_2H_5$	214	3.4	63
Prothion	$(C_2H_5O)_2P(S)SCH_2CH_2COOC_2H_5$	2600	104	25
Propoxon	$(C_2H_5O)_2P(O)SCH_2CH_2COOC_2H_5$	435	57	7.6
Methyl methprothion	$(C_2H_5O)_2P(S)SCH(CH_3)CH_2COOCH_3$	2000	60	33
cf. Malathion	 $(CH_3O)_2P(S)S\underset{CH_2COOC_2H_5}{\underset{\mid}{C}}HCOOC_2H_5$	815	12	68

[a] Data of O'Brien *et al.*[38]

On the basis of this hypothesis, several appropriate organophosphates were made.[28] As Table 9.3 shows, their selectivity was as predicted. Furthermore, the phosphates were always less selective than their phosphorothionate analogs, presumably due to the "opportunity factor." There should of course be other ways of utilizing the "opportunity factor": for example, phosphoramidates may in some cases be used instead of phosphorothionates.

The compound acethion (Table 9.3) was then examined further.[38] In one way the findings forced a modification of the hypothesis, for acethion was found to have a very low toxicity for the American cockroach: LD_{50} 1000 μg./gm. However, there was a good correlation between toxicity and *in vitro* metabolism: acethion was degraded readily by mouse liver slices and whole minced cockroach, and not at all by housefly mince. It was also shown that acethion was indeed degraded in mouse preparations by carboxyesterase action, so that the principal product was acethion acid, $(C_2H_5O)_2P(S)OCH_2COOH$.

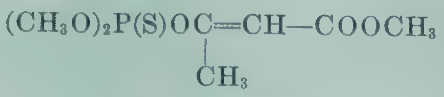
If carboxyester organophosphates are selectively toxic to insects, why is Phosdrin not selective?



$$(CH_3O)_2P(O)O\underset{CH_3}{\underset{\mid}{C}}{=}CH{-}COOCH_3$$

Phosdrin

It was possible that its (relative) nonselectivity was due to the lack of the "opportunity factor." If so, the thiono analog, "thiono-Phosdrin," should have selectivity.



$$(CH_3O)_2P(S)O\underset{CH_3}{\underset{\mid}{C}}{=}CH{-}COOCH_3$$

Thiono-Phosdrin

TABLE 9.4. TOXICITY OF PHOSDRIN AND THIONO-PHOSDRIN ISOMERS[a]

Compound	$\frac{LD_{50} \text{ mouse}}{LD_{50} \text{ housefly}}$
cis-Phosdrin	6
trans-Phosdrin	3
cis-Thiono-Phosdrin	3
trans-Thiono-Phosdrin	20

[a] Administered intraperitoneally to the mouse, topically to the housefly. Data are preliminary; precise data in Spencer.[46]

Now, both Phosdrin and thiono-Phosdrin have geometrical isomers, the *cis* and *trans* forms (page 58). The synthesis of a *cis*- and *trans*-thiono-Phosdrin was accomplished with considerable difficulty,[46] and the selectivity examined. As Table 9.4 shows, *cis*-thiono-Phosdrin was even less selective than *cis*-Phosdrin, but *trans*-thiono-Phosdrin was more selective than *trans*-Phosdrin. A hypothesis that would explain this oddity is that for geometrical reasons the *cis* isomers cannot be attacked by carboxyesterases, whereas the *trans* isomers can. This unwelcome additional hypothesis has not yet been confirmed by direct experiment, but it finds support in the observation[45] that the toxicity of *trans*-thiono-Phosdrin is potentiated by EPN, whereas the toxicity of the *cis*-thiono-Phosdrin and of the Phosdrin isomers is not. This is evidence (page 216) that carboxyesterase action is only important in the case of *trans*-thiono-Phosdrin detoxification.

Two modifications of the simple hypothesis of 1957 must, therefore, be introduced:

(*a*) From the acethion study, it is clear that organophosphates containing carboxyester groups are likely to show a restricted selective toxicity: the housefly conforms consistently with prediction, the American cockroach inconsistently. This difference may possibly be connected with the somewhat more vigorous carboxyesterase activity shown towards malathion by the American cockroach as compared with the housefly (Table 9.2).

(*b*) From the thiono-Phosdrin study, it seems that selectivity is not always conferred, even towards the housefly, by introducing a carboxyester group. The organophosphate must, for instance, have the right configuration to permit carboxyesterase attack.

In spite of these modifications, the writer has felt justified in applying to the carboxyester group the term "selectophore," defined as a group whose introduction into a toxic molecule confers selectivity of some kind.[32]

OTHER SELECTIVE ORGANOPHOSPHATES

The satisfactory accounting for malathion's selectivity on metabolic grounds prompted Krueger *et al.*[21] to extend this approach to other insecticides with low mammalian toxicity. Diazinon, dimethoate, and acethion were chosen, and parathion was also examined as a typical nonselective compound. This raises the question of definition, for parathion is, in fact, 6 times more toxic to the housefly or American cockroach than to the mouse. Tentatively, one might suggest a figure of twenty-fold in the difference of LD_{50}'s to A and to B before a compound can be called selectively toxic to one as compared with another. (This is a marginal figure: a fiftyfold difference should be the minimum aim in designing new low-hazard insecticides.)

The metabolism of the above four organophosphates was examined in the housefly, the American cockroach, and the mouse, *in vivo*. The nature of the degradation products was not established; only the variations of hydrolyzed and unhydrolyzed material and of the phosphates (i.e., the actual toxicants) were studied. The results for dimethoate and parathion are given in Fig. 9.1*b* and *c*. The most disturbing observation was that with parathion, which is nonselective, there was a marked difference between the levels of its phosphate analog, paraoxon, in insects and the mouse. The difference was somewhat like that noticed for malathion, (Fig. 9.1*a*) and was very similar indeed, to that noticed for dimethoate (Fig. 9.1*b*).

A detailed examination of the data of this and the preceding study suggested that the selective compounds showed two patterns of metabolism: malathion and Diazinon showed little difference in their chloroform-extractable levels (which may be taken as the levels of the parent compounds) between insects and mice, but a very marked difference in their phosphate analog levels, i.e., malaoxon and diazoxon. In contrast, dimethoate (Fig. 9.1*b*) and acethion showed very striking differences in their chloroform-extractable levels for insects as compared with mice, a difference much more extreme than that found in malathion or parathion; but the phosphate analog levels in insects and mice differed little more than in the case of parathion.

This suggestion of two patterns of metabolism was not made on the basis of qualitative examination of pictures such as Fig. 9.1, but on a roughly quantitative basis, illustrated in Table 9.5. Let us define the "P=O ratio" as the ratio of the concentrations of phosphate in the insect as compared to that in the mouse, and the "chloroform ratio" as the ratio of the concentrations of chloroform-extractable materials in the insect as compared to that in the mouse. Then the table shows that in the cockroach the P=O ratio for Diazinon (11.5) was far greater than that for parathion (3.0), but those for acethion (3.3) and dimethoate (2.3) were comparable to those for parathion. By contrast, Diazinon's chloroform ratio in the cockroach (1.2) was comparable to that for parathion (1.5), but dimethoate's was very much higher (11.0). At first it may seem discrepant that acethion's

TABLE 9.5. COMPARATIVE TOXICITIES AND LEVELS OF SOME METABOLITES IN INSECTS AND MICE 1 HOUR AFTER POISONING[a, b]

Insect	Ratio of levels or of toxicities			
	Parathion	Acethion	Diazinon	Dimethoate
Housefly				
$\frac{\text{LD}_{50}\text{ mouse}}{\text{LD}_{50}\text{ fly}}$	6	136	37	325
$\frac{\text{CHCl}_3\text{ fly}}{\text{CHCl}_3\text{ mouse}}$	1.6	5.6	1.1	7.7
$\frac{\text{P=O fly}}{\text{P=O mouse}}$	4.4	5.3	—	—
American cockroach				
$\frac{\text{LD}_{50}\text{ mouse}}{\text{LD}_{50}\text{ roach}}$	6	1.8	20	70
$\frac{\text{CHCl}_3\text{ roach}}{\text{CHCl}_3\text{ mouse}}$	1.5	2.2	1.2	11.0
$\frac{\text{P=O roach}}{\text{P=O fly}}$	3.0	3.3	11.5	2.3

[a] Data of Krueger *et al.*[21]

[b] $CHCl_3$ means the level of chloroform-extractable compounds. P=O means the level of the phosphate analog.

chloroform ratio in the cockroach was low (2.2) but this, in fact, was very fortunate: acethion is not toxic to the cockroach. In the housefly, to which it is toxic, the desired high chloroform ratio was found: 5.6 as compared to parathion's 1.6.

In summary of these elaborate findings: the metabolism of selective compounds differs from that of nonselective compounds in producing either higher chloroform ratios, as in acethion or dimethoate, or higher P=O ratios, as in Diazinon and malathion.

So much for observed results. What is the association between these results and the mechanism of selectivity? As far as an increased P=O ratio is concerned, the mechanism is evident: presumably, the difference in whole-body phosphate levels is reflected in the target area, and the target enzyme of the susceptible animal is, therefore, more inhibited. But when selectivity is associated with a high chloroform ratio, two possibilities exist. It may be that, in fact, the phosphate level *at the target* is higher in the susceptible animal, i.e., it does not reflect the over-all body level. Alternatively, if the target level does reflect the body level, it may be the greater persistence of a fairly low level of phosphate, caused by the persistence of the parent compound which is the phosphate's precursor.

The second alternative finds support from two very different findings. First, Vandekar and Heath[48] showed that the *in vivo* reversibility of the cholinesterase of organophosphate-poisoned mammals depended upon the persistence of the organophosphate in the body. Nonpersistent compounds which usually produced an inhibited enzyme which was readily reversible could be made to produce a relatively irreversible form if administered by slow infusion rather than in one dose. Secondly, Seume and O'Brien[44] and Knaak and O'Brien[16] concluded the EPN synergizes the toxic action of malathion in mammals not by inducing increases of malaoxon levels, but by causing persistence of malathion and, thereby, a low but persistent level of malaoxon at the target (page 216).

A final decision as to which of the alternatives explains the selectivity of compounds inducing high chloroform ratios must await further experimentation. Measurements of phosphate and phosphorothionate levels in regions of particular interest, such as the brain, may be useful in making such a decision.

It seems probable that for the four selective compounds discussed above (malathion, Diazinon, acethion, and dimethoate) selectivity is metabolic in origin. Only in the case of malathion is there direct evidence as to the nature of the metabolic difference in susceptible and nonsusceptible species, and in this case it is attributed to the high carboxyesterase activity of the nonsusceptible species. It seems highly likely that acethion owes its selectivity to the same cause, since mammalian tissues hydrolyze it at the carboxyester bond. Dimethoate is hydrolyzed in mammals primarily at the carboxyamide group, and it has been suggested by Dauterman *et al.*[10] that the selective toxicity of the compound to insects as opposed to mammals might be due to more vigorous carboxyamide hydrolysis in mammals.

$$(C_2H_5O)_2P(S)SCH_2C(O)\underset{\uparrow}{\,}OC_2H_5 \qquad\qquad (CH_3O)_2P(S)SCH_2C(O)\underset{\uparrow}{\,}NHCH_3$$

Acethion Dimethoate

But how can one account for differences in the degradation* of Diazinon? The limited data (page 220) indicate that Diazinon is degraded at the P—O bond, i.e., by phosphatase action.

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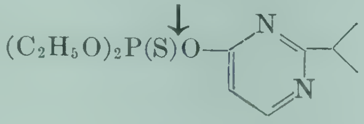
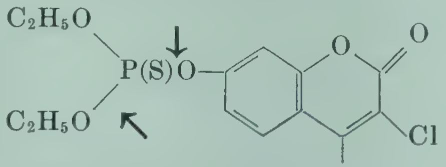
* Differences in P=S oxidation could account for selectivity, but the evidence is against this possibility;[21] for instance, the phosphate analogs of selectively toxic compounds are also selectively toxic, although to a lesser degree, due presumably to the lack of the opportunity factor.



Diazinon

And how can one account for the interesting pattern of the selectivity of Co-ral: it is very toxic to houseflies and cattle grubs, of intermediate toxicity to mice, and of low toxicity to cattle and rats? This pattern correlates well with the abilities of tissues from these animals to degrade Co-ral, the least susceptible having the most degrading activity.[39] It is known for the cow, rat, and goat *in vivo* that Co-ral, like Diazinon, is degraded by phosphatase action, in this case at two places:[19]



It is, therefore, quite likely that phosphatase activity for Diazinon and Co-ral is more vigorous in the mammal than in the insect. There are other insecticides of low mammalian toxicity whose degradations are probably due to phosphtase action: Delnav, Dipterex, ronnel, Chlorthion, and Morphothion are examples. It is, of course, premature to assume that their selectivity is metabolic in every case, but it is the writer's guess that this is true. Yet many other compounds which are degraded by phosphatases, such as parathion, Phosdrin, and Systox, show little selectivity. We may tentatively conclude that there are different phosphatases for these two classes, and that the phosphatases responsible for the degradation of the selective compounds are more effective in mammals than in insects. This is a hypothesis that urgently needs testing.

In a later section on the design of new selective compounds, further consideration will be given to the way in which metabolic differences can be used to obtain selective toxicity.

Penetration to the Target

So far, consideration has been given to the factors which control the level of actual toxicant in the body of a poisoned animal. However, only the fraction of that toxicant which arrives at the target is important in poisoning. We shall find that the amount which arrives at the target is a function not only of the concentration in the whole body, but, in some cases, of the physical properties of the toxicant and the permeability characteristics of the tissues which envelop the target.

The mammal has two important targets for phosphate attack: the central nervous system (C.N.S.) and the neuromuscular junction. The C.N.S. is substantially protected from ionized materials, the junction is not. By contrast, the insect seems to have only one target: its C.N.S., for the neuromuscular junction is apparently noncholinergic (page 170). The C.N.S. of most insects seems to be excellently protected from ionized compounds (page 162). The evidence for these statements is reviewed in Chapter 5.

The corollary of these facts should be that ionic cholinergic toxicants should be effective against mammals, attacking principally the neuromuscular junction, (since peripheral ganglionic effects are probably not lethal, at least in acute poisoning), but should be ineffective against most insects. This corollary was invoked in 1954 by O'Brien[26] to explain the apparent anomaly that acetylcholine was toxic to mammals but not to insects, in spite of the fact that both were thought to be killed by the excess *endogenous* acetylcholine produced in organophosphate poisoning.

An attempt has been made to utilize the hypothesis of an ion-impermeable barrier in insects to predict the toxicity to insects of ionizable compounds. The first approximation[29] was as follows: if a neurotoxic compound has an identical action in insects and mammals, and if insects but not mammals have their vital site protected completely from large ions, then all of an ionizable compound is effective in the mammal, but only the un-ionized fraction is effective in the insect. The size of the un-ionized fraction depends upon the pH, which we may assume to be about 7 for the insect body, and the pK_a of the compounds. Thus, for a base, the un-ionized fraction (F) is derived from the Henderson equation:[12]

$$\mathrm{pH} = \mathrm{p}K_a + \log_{10} \frac{u}{i}$$

where u is the molar concentration of the un-ionized form, and i is the molar concentration of the ionized form. Therefore,

$$F = \frac{u}{u + i} = \frac{1}{1 + \text{antilog}\,(\mathrm{p}K_a - \mathrm{pH})}$$

In the mammal, all of the compound is effective; in the insect only the fraction F is; therefore, the predicted toxicity ratio is

$$\frac{\mathrm{LD}_{50}\ \text{insect}}{\mathrm{LD}_{50}\ \text{mammal}} = \frac{1}{F}$$

In some cases, very acceptable agreement of prediction with observation was found: the observed and predicted LD_{50}'s were 7.7 and 11, respectively, for eserine, 9.1 and 10 for nicotine, and 3.4 and 2.6 for pilocarpine.[29] However, the observed figures had to be taken from the literature, and were from an assortment of species and techniques. An extensive study was therefore undertaken[34], using the mouse, injected intraperitoneally, as the reference mammal, and five insect species, injected into the hemocele. Thirty-five neurotoxic compounds were examined, representing ten different classes of pharmacological agents. The complex nature of the results necessitated a more elaborate analysis of expectations. Three conditions could exist. The term toxicity ratio will be used for (LD_{50} insect)/(LD_{50} mammal).

(*a*) If the agent was stable and had a cumulative action, an ion barrier should have no effect upon the compounds which were in equilibrium with an un-ionized form (i.e., most acids or bases, but excluding quaternary nitrogen compounds), for the ion barrier would only delay the time for equilibrium to be reached on each side of it; the un-ionized form would diffuse through, and form ions on the far side of the barrier. Then a toxicity ratio of 1 is expected.

(*b*) If the agent was very unstable (e.g., was rapidly detoxified or excreted) and had no cumulative action, the rate at which the toxicant reached the target would be the major factor. Since the rate of penetrating the ion barrier would be proportional to the concentration of un-ionized toxicant, the simple prediction, toxicity ratio $= 1/F$, would hold.

(*c*) If the agent was unstable but had a cumulative action, or if it was stable but had no cumulative action, the ion barrier would reduce the insect toxicity somewhat, and the toxicity ratio should lie between $1/F$ and 1.

We may add a fourth condition: if the ionized material had no un-ionized form with which to be in equilibrium, as is the case in quaternary nitrogen compounds, the compound should be nontoxic to insects; the toxicity ratio would be infinity.

The above arguments involve many simplifying assumptions of dubious validity, such as: that the mode of action is intrinsically identical in insects and mammals, and that the ion barrier is completely effective. Other factors interfere in obtaining appropriate "observed" data.[34] In order to test the above hypotheses, it is essential to have compounds in one class having a range of pK_a values, preferably from 5–9. Unfortunately, this is rarely possible. For organophosphates, only four quaternary nitrogen compounds and one sulfonium compound were tested, all unsuitable as far as pK_a is concerned. Since then, data on the base Amiton (page 163) has been obtained.[11] This compound has a pK_a of 8.4, and so a toxicity ratio of 25 was predicted. With the mouse as reference, the observed ratios were 220 for the American cockroach and >2000 for the housefly; these figures satisfy none of the above four conditions.

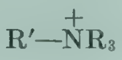
This indigestible bolus of theory is, at best, a nucleus for future experimental work. Few organophosphates were included in the study which accompanied the theory.[34] However, all of the ionic organophosphates studied so far show selective toxicity to mammals as compared with most insects; and with the exception of schradan, all the "selective mammalicides" are ionic or ionizable, as illustrated in Table 9.1.

Schradan is an interesting case of a compound which is not ionized, yet shows a toxicity pattern somewhat like that found with ionized organophosphates, such as Tetram,[4, 5] in being toxic primarily to mammals, aphids, and mites. This selectivity is not attributable to metabolic differences between susceptible and nonsusceptible species, for nonsusceptible insects produce from schradan, both *in vivo* and *in vitro*, ample anticholinesterase material to inhibit cholinesterase completely,[36, 37] yet the cholinesterase of treated insects remains uninhibited. This strongly suggests that the enzyme is protected in some way, and in fact O'Brien and Spencer[37] have shown, for the American cockroach, that if the nerve cord is damaged by cutting or stabbing, the anticholinesterase metabolite of schradan can reach the nerve cord cholinesterase. It is, therefore, tempting to speculate that the nonsusceptibility of the cockroach, and perhaps of other insects, is attributable to a barrier protecting the nervous system. Presumably, this barrier is lacking in susceptible arthropods.

It would economize in number of hypotheses if it were supposed that the insect nerve barriers to potassium (page 162), ionized toxicants, acetylcholine, and schradan are identical. Among these materials, schradan appears out of place, for it is not ionized. However, it seems significant that schradan, like the ionized organophosphates, does not inhibit brain cholinesterase of mammals *in vivo*. Assuming that this phenomenon is due to a failure of the active metabolite of schradan, hydroxymethyl schradan, to penetrate the blood-brain barrier, it follows that this metabolite, which is very polar,[47] behaves effectively as an ion as far as penetration is concerned. Even if this equivalence is allowed, however, the data in support of the notion of the identity of all these barriers are scanty; in some cases there are contrary data. For example, there is a considerable difference in the potassium permeability of the American cockroach and the locust nerve (page 169), yet both are unaffected by Tetram and acetylcholine. The milkweed bug and squash bug are poisoned by schradan, the American cockroach and housefly are not; but the responses of all four to ionized organophosphates are fairly similar.[33]

It is evident that ionization of organophosphates is of great importance in establishing a certain odd pattern of selective toxicity: a pattern in which susceptibility to ionized compounds is confined to mammals and a few arthropods (we know, of course, virtually nothing of the toxicities to animals other than mammals, insects, and mites). Thus, one generalization, not fully proven, might be that "ionic organophosphates may be toxic to mammals, aphids, and mites, but not to the majority of insects." We will be able to decide the validity of this statement, and be able to make other more extensive and useful ones, when we can answer the following question:

What is the relationship between the toxicities of ionizable and ionic compounds? It seems true for organophosphates[30] and carbamates[13, 17] that the quaternary compounds are much less toxic to insects than the tertiary, even though the tertiary would be mainly as an ionized species, e.g., with a pK_a of 9, a tertiary compound would be 99% ionized at pH 7. One would then have:



$R'\text{—}\overset{+}{N}R_3$

100% of quaternary form

$R'\text{—}\overset{H+}{N}R_2$

99% of tertiary form

These two forms should behave very much alike with respect to ion barriers and penetration of lipid materials. However, the similarity is somewhat deceptive. As far as the rate of penetration of a barrier is concerned, probably both may behave alike. But the equilibrium concentrations achieved will be very different. The argument on page 166 is applicable here: at physiological pH, the amount of the compound that will be in the nerve cord at equilibrium will depend upon the pK of the base and the partition coefficient of the un-ionized form. In the case of Tetram, the partition coefficient of the free base form (called Amiton) is probably quite in favor of a lipid phase.

$$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$$

Amiton

The pK_a of Amiton is 8.4;[30] if the partition coefficient were 100, then from Fig. 5.3 one sees that at pH 7 the concentration of Tetram in the nervous tissue would be four times that of the external concentration.

Quaternarized Amiton *does not have a free base form.* Quaternary nitrogen compounds cannot, under any pH conditions, take on the form R_4NOH, which would be the free base form (and is a form one encounters fairly often in textbooks!). Nitrogen cannot form five covalent bonds, although many attempts have been made to produce pentacovalent nitrogen compounds.[14] At equilibrium, therefore, no quaternarized Amiton can penetrate into very lipoidal tissue.

In the penetration of ionized materials there are thus two factors, one of which (the penetration rate) is similar for bases with high pK_a values and for quaternary compounds; the other (the equilibrium level attainable in a lipoidal tissue) of which presents little problem *at equilibrium* for most bases, as long as the partition coefficient of their free base forms are reasonably favorable, but which permits negligible entry of ionized compounds.

This argument accounts satisfactorily for the experimental observation of the dramatic differences between tertiary and quaternary nitrogen compounds. But the argument uses as a model a system in which the compounds are pictured as partitioning between aqueous and lipid phases. This is a much oversimplified picture. The neurons of all nervous systems are presumably accessible to numerous lipophobic compounds, such as the ions which are necessary for their metabolism and the nutrients which supply their energy needs. Presumably, these materials are supplied by the circulatory system. Why do lipophobic toxicants not penetrate by the same route? There is no information on this point. The problem may be purely one of rates. It is likely that detoxifying systems for organophosphates are quite widespread, so that a sufficiently rapid penetration of phosphate is needed so that these systems will be more than saturated.

The simple aqueous-lipid partitioning model does resemble, somewhat, the way in which materials in blood penetrate into the mammalian brain: phosphate equilibration requires 1 week; sodium equilibration requires 4–20 hours, compared with 20 minutes for muscle. But presumably glucose enters freely, and for a number of complex compounds such as sulfonamides, there is no correlation between penetration and liposolubility.[3]

In spite of the reservations necessitated by the tentative nature of the above discussion and data, the following working hypotheses may be proposed:

"Animals fall into two groups: group A comprises the mammals, mites, and aphids, with perhaps scales and various Hemiptera; group B comprises insects not in group A. Animals of group A can be killed as readily with ionized and ionizable organophosphates as with un-ionized. Animals of group B have low sensitivity to ionized compounds (particularly quaternary nitrogen compounds), and their susceptibility to an ionizable base will probably depend on the pK_a of the compound; the higher the pK_a, the less toxic will be the compound."

It seems that schradan and a limited number of the other phosphoramides behave like ionized compounds in that they are toxic to group A animals only. This may be attributable to their own physical properties or those of their active metabolites, which are liable to be more polar than their parents.

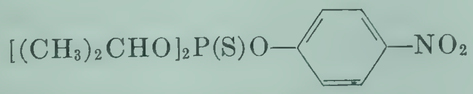
It would be interesting to know if acid neurotoxicants of low pK_a, which would thus be fully ionized at pH 7, would show lower toxicity for group B than group A animals. The hypothesis cannot readily be tested, for anionic organophosphates are liable to be poor anticholinesterases because of the nucleophilic effect of the anion upon the phosphorus. However, if the anion could be held away from the phosphorus so that neither inductive, field, nor mesomeric effects could be transmitted, an anionic anticholinesterase is a possibility. The permeability problem could, of course, be studied with tracer-labeled anions which need have no biological activity.

Nature of the Target

The evidence is fairly convincing that cholinesterase is the target for acute poisoning in mammals (page 177). In insects also, the cholinesterase hypothesis is the best one available, though it is not completely satisfactory. However, even this simplification does not allow us to discount the differences in the nature of the target. Table 3.8 indicates how much variation there is in the susceptibility of cholinesterases to organophosphates even among mammalian species. There appears to have been no attempt made to investigate whether these differences cause variations in the susceptibility to poisoning, as one would anticipate.

Differences between the enzymes of various arthropods and between arthropods and mammals would be expected. Casida[8] examined the variation in TEPP-susceptibility of acetylcholine hydrolysis in homogenates of 14 arthropod species, and in bovine acetylcholinesterase. He found that with aphids, mites, and wax moths TEPP (at $10^{-6}\,M$) had little effect, yet in the other species considerable inhibition was produced. Yet TEPP is a good aphicide and miticide,[25] so there is no correlation between the susceptibility of these arthropods and their cholinesterase.

A case of selectivity that has been attributed to differential enzyme susceptibility is that of diisopropyl *p*-nitrophenyl phosphorothionate:



In 1949, Metcalf and March[24] reported that it was 250 times as toxic to the housefly as to the bee, and was about 1000 times more potent *in vitro* against the cholinesterase of fly brain than of bee brain. This finding requires confirmation now that it is recognized that purified phosphorothionates have negligible *in vitro* anticholinesterase activity. It remains true that there is a dramatic difference between the cholinesterases of the fly and the bee, but it is uncertain for what compound the differential enzyme susceptibility exists. Probably it is an isomer of diisopropyl *p*-nitrophenyl phosphorothionate.

In vitro, but not *in vivo*, the cholinesterase of housefly head behaves quite differently from that of mouse brain or serum, in that it does not spontaneously reactive (page 101). This may be a peculiarity with no significance except *in vitro*, but it is possible that it represents a real difference between the insect and mammalian enzymes. If so, it should be possible to exploit such a difference to the disadvantage of the insect.

Attempts to examine the variations to be found in esterases of various species have been very limited. A recent extensive electrophoretic study by Augustinsson[2] of plasma esterases in 27 vertebrates, has revealed an astonishing variety of patterns. These enzymes are almost certainly not the target of poisoning, but one suspects that target enzymes may show similar variety in their properties.

So far, only the properties of the target enzyme have been discussed. Its location, too, may vary. The significant target in a mammal might be the respiratory center of the brain, the smooth muscle of the bronchi, or the neuromuscular junction of the chest muscles. The location of the target depends not only upon the organophosphate but also upon the species (page 176). Such differences could be put to use: thus, if a compound is equitoxic for two species, but attacks primarily the respiratory center in A and primarily the neuromuscular junction in B, then by adding a quaternary nitrogen (or other cationic) group to the compound its effectiveness against A will be reduced, whereas that against B will be affected very little (page 186).

Design of New Selective Compounds

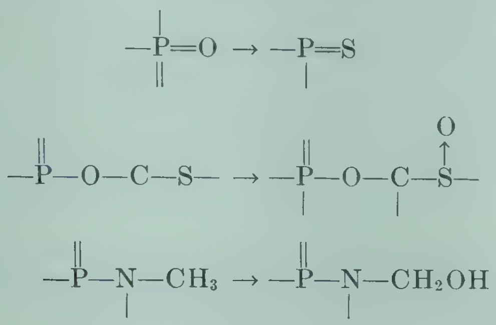
If we have a thorough understanding of how the organophosphates act, and why some are toxic only to certain species, it should be possible to design new organophosphates having predictable selective toxicity. Even with the imperfect knowledge we have now, it is possible to suggest the kinds of approach that may be profitable.

In the following discussion, the utilization of metabolic differences will be the only procedure suggested. However, the importance of ionization, as summarized on page 334, must be borne in mind, and will impose certain restrictions upon the permitted basicity of proposed new compounds.

In several cases it has been shown that an organophosphate kills animal A and not B because B has a highly active enzyme which can detoxify the organophosphate. It should, therefore, be possible to examine A and B for enzymic differences and utilize such differences to design new selective compounds. So far, the only published case where a remotely comparable attempt has been made to do this is in the case of the acethion group (page 324). It was made to test the hypothesis that vigorous carboxyesterase in B, the mouse in this case, should lead to the degradation of organophosphates containing carboxyesters. Even this application is made less exciting because acethion can be looked upon as an analog of malathion, and, therefore, could have been produced as a result of the familiar process of synthesizing all the compounds related to known effective insecticides.

What sort of differences can be utilized? There are two principles: one can arrange that the proposed susceptible species can activate the compound particularly well, or else that the proposed nonsusceptible species can degrade the compound particularly well.

Activation is a metabolic change which (in all cases known to date) makes the phosphorus more positive usually by making its substituents more electrophilic. The three best known activations, as described in Chapter 4, are all oxidative:



$$-\overset{|}{\underset{\|}{P}}{=}O \rightarrow -\underset{|}{P}{=}S$$

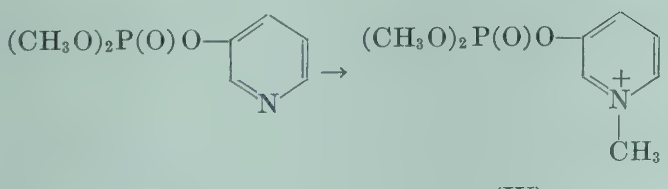
$$-\overset{\|}{P}-O-C-S- \rightarrow -\underset{|}{\overset{\|}{P}}-O-\underset{|}{C}-\overset{O}{\overset{\uparrow}{S}}-$$

$$-\overset{\|}{P}-\underset{|}{N}-CH_3 \rightarrow -\overset{\|}{P}-\underset{|}{N}-CH_2OH$$

There is no evidence to indicate that these oxidations differ very much in insects and in mammals: if they did, all phosphorothionates, thioethers, and phosphoramidates would be selective. Before rejecting the possibility of utilizing activating differences, other modes of activation might be investigated. Oxidation by monoamine oxidase is a possibility, e.g.,

$$\underset{\text{(I)}}{(CH_3O)_2P(O)OCH_2N(CH_3)_2} \rightarrow \underset{\text{(II)}}{(CH_3O)_2P(O)OCHO}$$

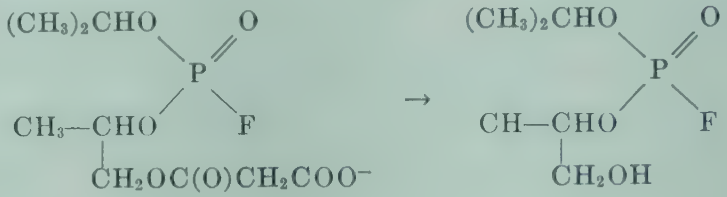
although in this case, (I) is probably a potent anticholinesterase even without activation, and further oxidation of the aldehyde of (II) is probable. Biological methylation, when it produces quaternarization, could be a potent activation mechanism, e.g.:



(III) (IV)

A comparable biological quaternarization of nicotinic acid occurs in dogs.[9] In the above hypothetical activation, one not only introduces a cation, which should make the phosphorus more positive, but also introduces a quaternary nitrogen at a very suitable place for binding to the anionic site of cholinesterase. Presumably, an animal which is particularly good at converting (III) to (IV) would be particularly susceptible to poisoning by (III). One suspects that (III) would be a selective mammalicide!

Another activation possibility is the removal of an anion by hydrolysis. This could be achieved by condensing one acid group of a dibasic acid to an alcoholic hydroxyl in a phosphate. In order to assure that the hydrolyzed phosphate residue will be insecticidal, it would be well to attach the hydroxyl to a known anticholinesterase, e.g., to DFP:

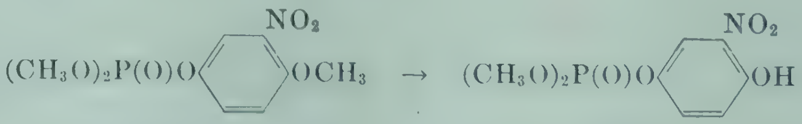


(V) (VI)

This suggestion involves the presupposition that the carboxylate ion of (V) can influence the positiveness of the phosphorus, presumably by a field effect (page 386), so that (V) is a poor anticholinesterase. It might be necessary to redesign (V) to improve this influence. Compound (V) should have possibilities as a plant systemic, at any rate for root application, by virtue of the water-solubility which the anion should bestow.

Let us now consider selectivity derived from differences in degradation, which may be considered as any reaction which makes the phosphorus less positive, usually by making its substituents more nucleophilic. The known cases involve the introduction of an anion by carboxyester hydrolysis, as in malathion and acethion, and we may tentatively include carboxyamide hydrolysis, as in dimethoate (page 328). Phosphate ester bond hydrolysis is, of course, almost universal in the organophosphates. The animal which carries out these hydrolyses vigorously should escape poisoning. Clearly, there is a huge field to be investigated in the introduction of ester and amide groups into organophosphates. In these cases (as in all cases of selectivity by degradation), the opportunity factor should be borne in mind (page 323) and, thus, phosphorothionates are the most promising candidates.

Carboxyesters are not the only esters whose hydrolysis can constitute a detoxification. Alkylated phenols can be hydrolyzed, and if the phenol is made acidic, for instance, by a nitro group, hydrolysis will yield a compound which is anionic at pH 7, e.g.,



(VII) (VIII)

It is important that the nitro group be included; thus, the pK_a of phenol is 9.95, so that at pH 7 the un-nitrated form of (VIII) would probably be substantially un-ionized, and therefore, hydrolysis of the un-nitrated form of (VII) at the methoxyphenol bond would not detoxify. The introduction of an *o*-nitro group into phenol brings the pK_a down to 7.2[15] so that (VIII) would be about half ionized at pH 7, and hydrolysis of (VII) would therefore introduce an anion and would be a detoxification.

There are other ways of introducing anions besides ester and amide hydrolysis. Some potential reactions are (where the end product is pictured in its form at pH 7)

$$—CH_2OH \rightarrow —COO^-$$

$$—CH_2CN \rightarrow —CH_2COO^-$$

$$—CHO \rightarrow —COO^-$$

If any of these reactions is particularly vigorous in one species, that species should be able to escape poisoning by an organophosphate containing one of the three groups.

An interesting detoxification mechanism has been described for parathion in which the ruminant escapes poisoning because of the reductive capacities of its microorganisms, which reduce parathion to aminoparathion (page 232). This dramatic phenomenon is due to the fact that the strongly electrophilic effect of the *p*-nitrophenol group is replaced by a marked nucleophilic effect of the *p*-aminophenol group (page 389). A similar but weaker effect should be found by reduction of a nitro group in aliphatic compounds, so that the following might, at first, be thought to be a detoxification reaction:

$$\underset{\text{(IX)}}{(CH_3O)_2P(O)OCH_2CH_2NO_2} \rightarrow \underset{\text{(X)}}{(CH_3O)_2P(O)OCH_2CH_2NH_2}$$

However, there are two complicating factors. In the first place, the NH_2 group in aliphatic compounds displays a weak electrophilic inductive effect (page 383) in place of the strongly nucleophilic mesomeric effect it shows in aromatic compounds. Therefore, replacing the nitro group in (IX) by an amino group gives only a moderate reduction in the positiveness of the phosphorus, i.e., due to replacing a strong electrophile with a weaker one. Even more important, the amino group in aminoparathion is a weak base, its pK_a, presumably, being similar to that of aniline: about 4.3. Consequently, it does not have a protonated form at pH 7. But with compound X we have a different situation: X is close enough to Tetram to suggest a pK_a of about 8.5 to 9, consequently, most of X would, at pH 7, have the protonated form:

$$(CH_3O)_2P(O)OCH_2CH_2\overset{+}{N}H_3$$

The protonated group will help binding to the anionic site of cholinesterase, as has been shown for Tetram.[30] Also the $^+NH_3$ behaves as quite a strong electrophilic group. It is one of the interesting properties of amines that depending upon pH and pK_a, they can exist as the NH_2 form, which is weakly electrophilic in unconjugated systems, or the $^{+}NH_3$ form, which is strongly electrophilic in such systems. It is in fact likely that $-{}^{+}NH_3$ will be at least as electrophilic as, and perhaps more electrophilic than, $-NO_2$, which we can more accurately represent as

$$-\overset{+}{N}\overset{-}{O_2}$$

Summarizing the situation with regard to the reduction of (IX) to (X), it is quite probable that the reduction may constitute an activation rather than a degradation. The case serves to illustrate how many complicating factors must be considered in predicting the modification of properties to be expected as a result of a given reaction.

Since virtually any modification of a molecule will alter the electrophilic properties of some part of it, it follows that the metabolic modification of an organophosphate will virtually always constitute either an activation or a degradation. Furthermore, as Appendix 2 shows, there is little restriction upon the nature of the side group which can be attached to a toxophore such as $(RO)_2P(S)S-$, as long as the actual toxicant produced in the susceptible animal is a potent anticholinesterase. The factors necessary to meet this requirement are discussed on pages 83–96. One has, therefore, great freedom in designing side groups. Where there is a reaction which is not highly specific, and whose extent varies among different species, it should be possible to utilize the variation to obtain selective toxicity.

These considerations may be formulated into a generalization, of which the reactions discussed above are specific examples. When the metabolic step, $A-B \rightarrow A-C$, occurs more in species X than in Y, and B is substantially more electrophilic than C, and A can be any of a large number of substituents, then the reaction can be used to obtain selective toxicity towards Y as compared with X, for if A is an $(RO)_2P(O)O$ group, the reaction will be a detoxification. If, on the other hand, C is substantially more electrophilic than B, the reaction, when A is an $(RO)_2P(O)O$ group, will be an activation, and the compound will be selectively toxic to X as compared with Y.

The above argument must be modified by consideration: (*a*) of the opportunity factor—if the detoxification principle is to be utilized, an opportunity for detoxification must be allowed, e.g., by making $(RO)_2P(S)OX$ rather than $(RO)_2P(O)OX$; (*b*) of the effect noted on page 92, that beyond a certain point, additional electrophilic substituents may worsen rather than improve anticholinesterase activity because the organophosphate becomes too unstable; (*c*) of the ionization phenomenon, whereby an ionizable organophosphate may have a peculiar species specificity (page 334); and (*d*) of steric phenomena. It is to be expected that certain attachments to the toxophore may hinder the ability of the phosphorus to attack cholinesterase. However, extraordinarily bulky groups have been successfully attached, as in Co-ral, Guthion, and Bayer 22408.

How should the above principles be applied, and what sort of body of knowledge can we hope to derive from their application?

There are two facets to the application problem. First, one must find out what differences exist between the animals to be spared and those to be killed, then he must decide how to utilize the differences in the design of new compounds. The first part should not be approached only by studying differences in the metabolism of organophosphates. Other simpler substrates should be tested. Once a given difference has been established, it will be necessary to determine the specificity of the enzyme responsible for the difference. If it is not highly specific, it may form the basis for a selectophore to be built into a known toxophoric nucleus.*

It will be necessary to examine more closely the requirements for a good toxophoric nucleus. Selective and safe insecticides are of little use unless they have excellent insect toxicity. Even though compound X has a toxicity ratio (insects to mammals) of 500 and Y has a ratio of 100, Y may still be the better compound if it has an LD_{50} to insects of 2 μg./gm. compared to X's LD_{50} of 10 μg./gm. A fivefold difference of insect LD_{50} can mean the difference between economic usefulness and failure. The design of a new selective compound should, therefore, start with a compound with extreme toxicity for all animals, and selectivity can then be introduced by adding a selectophore. It seems probable from past experience that a selectophore will commonly reduce the toxicity somewhat even to the susceptible animal.

It should be possible, if an extensive enough search is made, to develop a set of principles of organophosphate selectivity. Perhaps the carboxyester principle (page 323), the principle of the opportunity factor (page 323), and the ionization principle (page 334) may be considered as extremely crude attempts at such a set. We must expect that most of the principles will be more complex than these, and probably more subtle. Basically, they will consist of a series of ways of classifying animals into two groups, susceptible and nonsusceptible; there would be a different way for each class of selectophore. Sometimes two species will be together in one class, sometimes in another, sometimes they will be in different classes. Clearly, such classifications will depend upon a great extension of the number of species to be examined in detail: at the moment, houseflies, cockroaches, rats, mice, and cows are the only animals which have received serious study.

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* Because the terms "selectophore" and "toxophore" can be conveniently applied to different parts of an organophosphorus molecule does not imply that they are without interaction. The anticholinesterase activity of an organophosphate is a function of all the substituents on it which modify either its steric suitability or the positivity of its phosphorus. Similarly, the selectophoric portion may well be effective when attached to some toxophores but not to others.

Besides developing modes of classifying important species, it is to be hoped that in the course of this development there will develop a series of simple *in vitro* biochemical tests, such as the oxidative, reductive, and hydrolytic behavior of whole homogenates, which can be applied rapidly to any new arthropod which may in the future achieve the status of an economic pest. Nor should we restrict our consideration to arthropod pests. There is no basic reason why selectivity between mammalian species should not be achieved: safe organophosphates for rodent control are quite within the bounds of possibility.

In 1951, Professor Albert[1] wrote: "No research institution exists anywhere in the world for the study of the principles of selective toxicity, and existing institutions are not yet devoting any considerable portions of their funds to such studies which require the organized co-operation of physical and organic chemists and many categories of biologists." In almost ten years, the situation has changed but little. Let us hope that the next ten years will demonstrate that this kind of study is not only possible but can produce rich dividends in useful pesticides as well as in the understanding of the basic life processes and their disruption.

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CHAPTER 10

Techniques

A chapter on the subject of techniques may seem out of place in a book of this nature. In particular, I have had doubts about including a few details on synthetic procedures when I have not discussed the organic chemistry of phosphorus compounds in previous chapters. My reasons are as follows.

The field of organophosphate biochemistry has always been limited by available techniques. For instance, only when it became apparent that the average phosphorothionate was contaminated with isomers which were more active *in vitro* than their parent compounds was any real insight into the mode of action of these compounds possible. Then again, when radioactive materials were not easy to obtain or use, many lengthy and only partially conclusive studies (my own included) were made, which could have yielded far more information with less work if organophosphates of high specific radioactivity had been used. Almost every study upon organophosphate metabolism *in vivo* should be done with labeled compounds.

The greater part of the work described in this book has been done in laboratories having rather simple equipment for organic chemistry. Yet studies with labeled compounds are enormously expensive unless one can synthesize his own material, and the problem of purification techniques introduces a hundred pitfalls in the path of one who takes on trust the compounds given to him. Fortunately, methods have been developed during the last few years which should enable anyone with a working laboratory knowledge of organic chemistry to prepare and purify many of his own compounds, labeled or "cold." In particular, some of the techniques described have been designed for use with the very small quantities of radioactive material with high specific activity which are particularly necessary in work on insect metabolism. These techniques are suitable for use in (for instance) entomological laboratories, where esoteric equipment for chemistry is simply not available.

As for other techniques, editorial limitations in original research papers often result in the "methods" section being sandwiched, in humiliating small print, between the "introduction" and "results," and so abbreviated that the novice is at a loss to understand it. When the author is allowed to state his method, he usually does not have room to state why he used his method in particular, or to mention the five other methods which did not work. It is hoped that this chapter will give a plain account of some of the methods of particular use in studies of organophosphates, in sufficient detail to allow any competent scientist to duplicate them and know what to avoid.

I am greatly indebted to Drs. E. Y. Spencer and W. C. Dauterman for guidance in preparing this chapter. Many of the techniques are those developed in Dr. J. E. Casida's laboratory.

Radio Tracer Synthesis

This section will deal with procedures which are simple, and give materials (often in low yield) which are pure and of high enough specific activity to make it possible to study their metabolism by insects. Full details will not be given when they have been published already.

Nobody should attempt these syntheses without some knowledge of the hazards, precautions, and special techniques which are needed in handling radioactive materials. A useful book on safety aspects is that by Boursnell.[13] Comar's book[22] gives a more detailed account of techniques and principles, with particular emphasis on use rather than syntheses. A special factor in P^{32} syntheses is the need for scrupulous dryness of reagents (particularly organic solvents, which should be specially dried) and apparatus both before and during reactions: all condensors, for instance, should be fitted with drying tubes. All the reactions should be performed in the fumehood, with ground-glass jointed apparatus.

The source book for generalized procedures, and for references to more detailed ones, is Kosolapoff's "Organophosphorus Compounds";[57] unfortunately, it only covers publications up to 1949. Many useful syntheses are described in Metcalf's "Organic Insecticides,"[71] published in 1955.

The phases involved are: (*a*) exchange, whereby the radioactivity purchased is transferred to the starting material; (*b*) synthesis of a phosphorus intermediate such as $(RO)_2P(S)SH$; (*c*) coupling the intermediate with the appropriate compound to yield the product; and (*d*) purification.

EXCHANGE

There are four phosphorus starting materials from which almost all the currently used organophosphates can be prepared. They are:

(*1*) PCl_3 and $P(O)Cl_3$, from which phosphates or phosphorothiolates can be made. For nearly all subsequent syntheses except that of phosphoramides, PCl_3 gives by far the best yield, and so is preferred.

(*2*) $P(S)Cl_3$, from which phosphorothionates can be made.

(*3*) P_2S_5, from which phosphorodithioates can be made.

In order to made whatever compounds one wishes it is, therefore, necessary to have methods for preparing the above compounds.

The early techniques used irradiated red phosphorus as source. This is not a convenient material to handle, nor can high-activity intermediates be obtained from it. A better source is $H_3P^{32}O_4$, which is the standard pure P^{32} preparation of high specific activity obtainable from atomic energy establishments. In 1940, Chargaff and Keston[20] made $P^{32}OCl_3$ by the reaction:

$$Ag_3PO_4 + PCl_5 = 4POCl_3 + 3AgCl$$

The reactants were heated at 130°C. for 10 minutes. This basic procedure was modified in 1954 by Kalinsky and Weinstein[51] who used $H_3P^{32}O_4$, dried it carefully, then reacted it with PCl_5, first in the cold, and then under reflux.

Vigne *et al.*,[106] in 1956, used another modification: $Na_3P^{32}O_4$ and Ag_3PO_4 were dried and heated with PCl_5 in a sealed tube at 140°C. for 30 minutes, to give $P^{32}OCl_3$ as above (this was subsequently heated with sulfur at 160°C. for 2 hours to give $P^{32}SCl_3$).

A new type of procedure was utilized by Vigne and Tabeau[107] in 1958: the P^{32} was directly exchanged with P^{31}, e.g.,

$$H_3P^{32}O_4 + POCl_3 = H_3PO_4 + P^{32}OCl_3$$

This apparently worked for $POCl_3$, $PSCl_3$, and PCl_3. The way in which we have used this procedure for $PSCl_3$ is as follows.[27]

The desired volume of $H_3P^{32}O_4$ in dilute HCl (i.e., as received from the supplier) is pipetted into a Carius tube about 6 inches × ½ inch which has been drawn out and thus constricted about 1 inch from its open end. The water and HCl are removed by blowing dry air into it through a long hypodermic needle. The $PSCl_3$ is added, and the tube is cooled in acetone-dry ice to prevent loss during sealing; then it is sealed at its constriction in an oxy-gas flame; the seal must be perfect. The tube is held in a piece of iron tubing (in case of explosion) at the temperature shown below, using a small muffle furnace; then it is allowed to cool and is opened. The resultant labeled compound, which can be used without purification, is washed out into the reaction vessel with the desired organic solvent.

The temperatures and times (which can undoubtedly be improved) are:[107]

$POCl_3$: 90% exchange in 8 hours, 150°C.

$PSCl_3$: 68% exchange in 60 hours, 150°C.

PCl_3: 65% exchange in 14 hours, 150°C.

It is very probable that at higher temperatures, better exchange could be obtained.

Also in 1958, Casida[16] reported that in various procedures the exchange with $PSCl_3$ was poor, but that with P_2S_5 the exchange was almost complete.

He placed the P_2S_5 with $Na_3P^{32}O_4$ or $H_3P^{32}O_4$ in a flask fitted with a condensor, gassed the system with carbon dioxide, and heated by flame, first gently, and then to boiling for 10 minutes. The reaction flask could be used to carry out subsequent steps of the total synthesis, so that transfers were in some cases completely eliminated. This is very important when working with radioactive material. He also found that heating the P_2S_5 with the $H_3P^{32}O_4$ in ampules at 300–500°C. was effective.

Casida described a step by which this $P^{32}S_5$ could be converted to $P^{32}SCl_3$. To the $P^{32}S_5$ was added PCl_5 and very dry $AlCl_3$, the flask was fitted with an air condensor and the system was sealed with glass stoppers held in by springs. The flask was heated for 90 minutes at 160°C. in an oil bath, the stoppers being jammed home once the temperature was attained. A disadvantage of this method (compared to a direct exchange, if indeed it is efficient with $PSCl_3$) is that it dilutes the P^{32} with unlabeled phosphorus, in the ratio 3 of unlabeled phosphorus to 2 of P^{32}, and one thereby loses specific activity.

$$P_2S_5 + 3PCl_5 = 5PSCl_3$$

As to which method to select, it is the writer's unsubstantiated view that Casida's flask method is best for P_2S_5, whereas Vigne and Tabeau's tube method is best for $POCl_3$, $PSCl_3$, and PCl_3. But a thorough study of the time-temperature-yield relations for both procedures is needed before a reliable statement can be made.

PREPARATION OF THE INTERMEDIATE

Next comes the problem of converting the starting material to (usually) the dialkylated intermediate, such as $(RO)_2P(S)SH$. The techniques are described in detail by Kosolapoff.[57] Basically the high-yielding routes are as follows.

(*a*) *For phosphorodithioates:*

$$4ROH + P_2S_5 = 2(RO)_2P(S)SH + H_2S$$

The calculated volume of alcohol dissolved in toluene is slowly added to the P_2S_5 suspended in toluene, and the mixture is refluxed until the P_2S_5 is gone (about 90 minutes).

(*b*) *For phosphates and phosphorothiolates:*

(i) The most efficient way is a two-step method. To the PCl_3 in chilled benzene is added a calculated volume of alcohol, and the system is refluxed for 30 minutes.

$$3ROH + PCl_3 = P(OR)_3 \qquad \text{(yield about 93\%)}$$

Then chlorine is bubbled in until the solution is yellow, then the excess is blown off by bubbling air through for 15 minutes.

$$P(OR)_3 + Cl_2 = (RO)_2P(O)Cl + RCl \qquad \text{(yield about 87\%)}$$

(ii) A low-yielding but simpler alternative is to add the alcohol to $POCl_3$:

$$2ROH + POCl_3 = (RO)_2P(O)Cl + 2HCl$$

The calculated volume of alcohol is added dropwise over 30 minutes to the $POCl_3$ in benzene, at room temperature. This reaction is probably capable of considerable improvement in yield if the HCl is removed, e.g., by reaction in pyridine, the pyridine-HCl bing filtered off later.

A simpler variant would be:

$$2RONa + POCl_3 = (RO)_2POCl + 2NaCl$$

(*c*) *For phosphorothionates and some phosphorodithioates:*

(i) The $(RO)_2P(S)SH$ prepared as in (*a*) is chlorinated in benzene.

$$2(RO)_2P(S)SH + 3Cl_2 = 2(RO)_2P(S)Cl + S_2Cl_2 + 2HCl \qquad \text{(yield about 50–75\%)}$$

The temperature should be kept below 20°C, by an ice bath if necessary, and the chlorine passed in slowly. For small amounts, this may be more easily done by adding benzene containing a known amount of chlorine. This original method[3] has been improved[68] by the use of methylene chloride as solvent, and pyridine to remove the HCl. Another method uses gaseous HCl in the presence of benzonitrile for the chlorination.[110] Washing with sodium sulfite after chlorination has been recommended.[30] None of these improvements have been studied under conditions of tracer synthesis; undoubtedly the original method can be improved.

(ii) A simpler alternative, involving only one step, is often convenient:

$$2RONa + P(S)Cl_3 = (RO)_2P(S)Cl \qquad \text{(yield about 50\%)}$$

The $P(S)Cl_3$ in benzene is placed into a flask fitted with a condensor, stirrer, and dropping funnel, and then is placed into an ice bath. The calculated amount of sodium alcoholate in excess alcohol is added dropwise with stirring over 15 minutes, then stirring is continued for 15 minutes.

(*d*) *For phosphorodiamidates:*

A useful intermediate is $(R_2N)_2POCl$. In the case of the dimethylamino compound, this can be made in two ways,[35] the most convenient for tracer work being:

$$POCl_3 + 4NH(CH_3)_2 = [(CH_3)_2N]_2POCl + 2NHCH_3 \cdot HCl$$

The $POCl_3$ in ether is cooled in dry-ice alcohol and is stirred by nitrogen. The dimethylamine is added slowly, then the system is allowed to come to room temperature and stirred for another 12–15 hours. Filtration removes the amine hydrochloride, and the ether is easily removed under vacuum.

COUPLING

The final step is to couple the intermediate with the side chain X. The details are described in the literature, either in the first publications concerning the compound or in the patent. Either of these can be obtained through the subject or formula index of *Chemical Abstracts*.

Typical routes are as follows:

(*a*) *Phosphorodithioates:* Made by coupling the halogenated X-group with the potassium, ammonium, or other salt of $(RO)_2P(S)SH$. Thus, for acethion:

$$(C_2H_5O)_2P(S)SK + ClCH_2COOC_2H_5 = (C_2H_5O)_2P(S)SCH_2COOC_2H_5 + KCl$$

(Chloroethyl acetate)

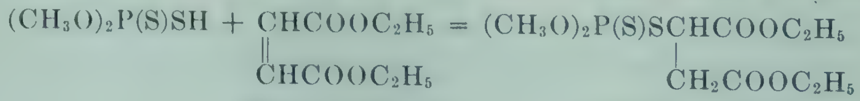
In nonradioactive syntheses, it is easy to prepare the potassium salt, $(RO)_2P(S)SK$ from $(RO)_2P(S)SH$, by dissolving it in acetone, and refluxing it while adding the XCl, continuing the reflux for (say) 12 hours. However, for radioactive material, isolation of the salt is inconvenient. Two alternatives have been used.

(1) To the solution of $(RO)_2P(S)SH$ in (say) benzene is added a calculated amount of KOH, the mixture is shaken and the benzene removed; the aqueous phase now contains $(RO)_2P(S)SK$ and is added dropwise to CXl in refluxing acetone. After refluxing (e.g., 18 hours) the KCl is filtered off, the acetone is largely removed by an air stream, and the $(RO)_2P(S)SX$ is extracted from the water by chloroform.

A better procedure, with less probability of hydrolysis in this prolonged heating is the following one.

(2) Ammonia is bubbled into the $(RO)_2P(S)SH$ in benzene, and the ammonium salt precipitates. Excess ammonia is blown off by an air stream, and the XCl is added in an organic solvent. After refluxing (e.g., 6 hours) the NH_4Cl is filtered off.[97]

Another principle (not very common, but very easy when it can be applied) is substitution across certain double bonds. Thus for malathion:

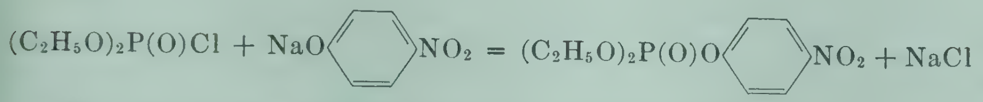


$$(CH_3O)_2P(S)SH + \begin{array}{l} CHCOOC_2H_5 \\ \| \\ CHCOOC_2H_5 \end{array} = (CH_3O)_2P(S)S\begin{array}{l} CHCOOC_2H_5 \\ | \\ CH_2COOC_2H_5 \end{array}$$

Diethyl maleate Malathion

The diethyl maleate is added to the $(CH_3O)_2P(S)SH$ in toluene and refluxed for 4 hours.

(*b*) *Phosphates:* Also made by eliminating KCl, NaCl, or NH_4Cl, but in this case the K, Na, or NH_4 in the starting material is on the side chain X. Thus, for paraoxon:



(yield about 60%)

The diethyl phosphoryl chloride is added slowly to the sodium *p*-nitrophenate in refluxing acetone or dioxane, and refluxing is continued for several hours.

An alternative approach is:

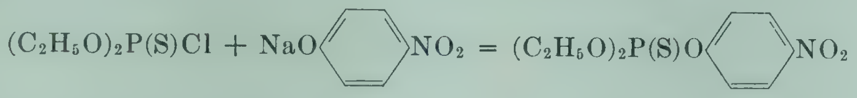
$$XH + P(O)Cl_3 = XP(O)Cl_2 + HCl$$

$$XP(O)Cl_2 + 2NaOR = (RO)_2P(O)X + 2NaCl$$

but this is a more difficult and less efficient method.

Another procedure uses the free XH along with Na_2CO_3, in place of NaX. The mixture is refluxed in acetone for 5 hours.

(*c*) *Phosphorothionates:* The procedures are those described in (*b*) except that $(RO)_2P(S)Cl$ replaces $(RO)_2P(O)Cl$. Thus, for parathion:



The other alternatives cited in (*b*) are also applicable.

(*d*) *Phosphorothiolates* (*and some phosphorodithioates*)*:* The same principle is used as in (*b*), but instead of sodium alcoholate, one uses a sodium mercaptoalcoholate, NaSX. For phosphorothiolates, this is coupled with $(RO)_2P(O)Cl$; for phosphorodithioates it is coupled with $(RO)_2P(S)Cl$. Thus, for Amiton:

$$(C_2H_5O)_2P(O)Cl + NaSCH_2CH_2N(C_2H_5)_2$$
$$= (C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2 + NaCl$$

To the sodium mercaptoalcoholate in refluxing acetone is added, dropwise, the $(RO)_2P(O)Cl$, and the mixture is refluxed for 4–5 hours. The NaCl precipitates, the product in acetone is filtered off.

(*e*) *Phosphorodiamidates:* A useful generalized procedure (which has been used for schradan and dimefox) uses KX or NaX:

$$[(CH_3)_2N]_2POCl + KX = [(CH_3)_2N]_2POX + NaCl$$

In the case of dimefox,[88] the KF was added in aqueous solution to the $[(CH_3)_2N]POCl$ preparation in chloroform, and the mixture was stirred for 15 minutes without heating or cooling. For schradan, an analogous preparation has been suggested[21] but it appears less adapted to tracer synthesis[35] than:

$$\underset{\text{(I)}}{[(CH_3)_2N]_2POCl} + \underset{\text{(II)}}{[(CH_3)_2N]_2P(O)OC_2H_5} = [(CH_3)_2N]_2P(O)OP(O)[N(CH_3)_2] + C_2H_5Cl$$

Compound (II) is prepared by adding sodium ethoxide, slowly, to (I), refluxing for 20 minutes, and filtering off NaCl. Then (II) is reacted with more (I) in xylene, refluxing for 20 hours.

PURIFICATION

The first step is to separate the product from the gross mixture of side products and unreacted parent material. A common procedure is to wash the mixture, by adding water to the organic solvent which usually contains the mixture, shaking in a separatory funnel, and discarding the aqueous phase. This is repeated twice more. In place of water, 5% Na_2CO_3 is often used for the first two washings, in order to hydrolyze polyphosphates and phosphoryl halides, and to keep the acid intermediates completely ionized so that they are removed completely from the organic solvent phase. Although this procedure is usually effective for phosphorothionates, several phosphates are too unstable in alkali, and water-washing should be substituted.

After washing, the solvent containing the product is dried, usually with Na_2SO_4, and the solvent is removed. For solvent removal, a rotary evaporator is almost essential: good commercial ones are available (e.g., Labline or Rinco), and details for homemade ones have been published.[56, 111] Solvent removal under air blast is slower, and more likely to introduce contaminants and disperse radioactivity.

Subsequent purification is classically performed by vacuum distillation, for which an oil pump is needed to obtain pressures of the order of 0.05 mm. of mercury: high distillation temperatures should be avoided. However, with the small quantities of product, such as 0.2 to 1 gm., which are often prepared for tracer work, the usual distillation techniques are impossible because of losses on flask walls, condensors, and so on. Column purifications are useful in such cases. For this purpose Celite, silica gel, or alumina columns may be used:[5, 17, 18, 28] the suitability of each and the details of their use are described below in reference to their analytical use. In radioactive work, the location of the principal peak of activity (presumably the product) may often be found simply by placing each fraction at a fixed distance from a survey meter.

The major peak should preferably be divided into a few fractions and some criterion of purity applied to each. If an instrument is available, infrared spectra on each fraction may be sufficient. Simpler procedures might be to find the refractive index of each: those cuts whose index differs from that of the central cut may be discarded or purified with another column. If no such analytical procedures are available, the center cut may be taken and used, but some kind of check should be run, e.g., an anticholinesterase assay or even an LD$_{50}$ against a small insect (so that little is used up in the assay).

PRODUCT

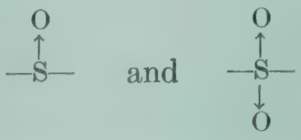
What sort of activities are to be expected from the syntheses described above? In the following calculations, decay losses will be neglected. Typically, one might start with 20 mc. of P^{32}, and prepare about 0.5 gm. of product. If the efficiency of the exchange was 65%, the starting material (e.g., PCl$_3$) would have 13 mc., and if this was converted to an insecticide with an efficiency of 60%, the 0.5 gm. of product would have 7.8 mc. (this activity should not be much lessened during purification). One mc. gives 3.7×11^7 disintegrations per second. In a typical counting apparatus of 15% efficiency (i.e., in which 15% of the disintegrations were counted) the product would have an activity of $7.8 \times 3.7 \times 11^7 \times 60 \times 15/100 \times 1/500{,}000 = 5200$ c.p.m. per μg. One could, therefore, easily measure 0.01 μg. Suppose 1 gm. of flies was treated with 10 μg./gm. of insecticide, and a given metabolite was eventually distributed over five countable fractions from, for instance, a chromatogram. Then, if this metabolite constituted 1% of the dose, the average activity from the five fractions (if the whole fraction could be counted) would be $1/5 \times 1/100 \times 5200 \times 10 = 104$ c.p.m., which can be measured fairly readily. The metabolism of such an insecticide could, therefore, be studied with some precision, with little risk of missing any but the most insignificant metabolite, and excellent quantitation for the important metabolites could be obtained.

Analysis

EXTRACTION OF METABOLITES FROM HOMOGENATES

Metabolites of organophosphates usually fall into two well-defined classes:

(*a*) Metabolites extractable from aqueous systems by solvents such as chloroform, hexane, benzene, etc. (In this group are the parent compounds, their oxidation or reduction products, e.g., their P=O analogs, and their sulfoxide and sulfone products,



$$-\overset{\overset{\displaystyle O}{\uparrow}}{S}- \quad \text{and} \quad -\underset{\underset{\displaystyle O}{\downarrow}}{\overset{\overset{\displaystyle O}{\uparrow}}{S}}-$$

and in the case of nitrophenol compounds their aminophenol analogs.)

(*b*) Common hydrolysis products, which are anionic at pH 7, e.g., $(RO)_2P(O)SH$, $(RO)(HO)POX$.

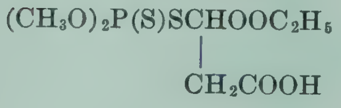
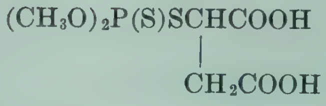
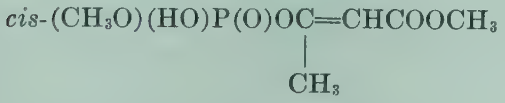
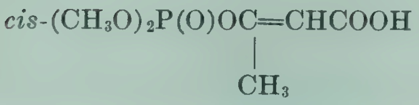
The above classes are often rather inaccurately called (say) the "chloroform-solubles" and the "water-solubles" respectively. This is not a desirable nomenclature; for instance, schradan is chloroform-extractable, but is also extremely water-soluble.

The great majority of metabolites behave as just described. It is important to note, however, that the separation is based upon the ionic character of the hydrolysis products. Now in fact this character depends upon the pH of the system as well as the compound. The products of phosphatase action [e.g., $(C_2H_5O)_2P(S)SH$] have a very low pK, that is to say, they are substantially ionized except under very acid conditions. The products of carboxyesterase and carboxyamidase action [e.g., $(C_2H_5O)_2P(O)SCH_2COOH$] have a considerably higher pK, in the region of 3.5, and therefore will only be solvent-unextractable at a pH substantially above 3.5. Where one desires to leave all the hydrolysis products in the aqueous phase, it is important to neutralize before extraction if there is the possibility of such a compound being present. Where it happens to be convenient to make an acid extraction first (see below) one should, back-extract the solvent phase with a neutral aqueous phase (e.g., buffer at pH 8) to remove carboxyesterase products; the two aqueous extracts are then combined.

Table 10.1 gives some representative pK values. In general, one expects that acids will be extracted readily into organic solvent if the pH is well below its pK. Unhappily this is not always the case: the carboxyacid derivatives of organophosphates are commonly chloroform-unextractable even from strong acid. They can often be extracted with ether, however. Fortunately, the reverse situation is rare: it seems that any acid metabolite is poorly extracted if the pH is adjusted to 2 or 3 units above the pK. In theory, however, one would expect that exhaustive extraction would extract acids into organic solvents even at a pH well above the pK, if the partition coefficient of the un-ionized acid is in favor of the organic solvent. The reasoning is precisely that discussed for base extraction into lipids (page 166 and Fig. 5.3).

A few exceptions exist to the rule that hydrolysis products are un-extractable, unhydrolyzed metabolites are not. If the hydrolysis should yield a nonionic product (e.g., in the hydrolysis of acetyl Dipterex to Dipterex) this product will probably be solvent-extractable. If the parent compound is ionic (e.g., quaternarized Tetram) of course it will be solvent unextractable. If the parent is basic, its extractability will vary with pH, since it will be 99% ionized at 2 units below its pK_a, and 99% un-ionized at 2 units above the pK (for example, Tetram is extracted well at pH 10, poorly at pH 5). Finally, there are some ionic rearrangement products, as mentioned on page 61.

TABLE 10.1. pK_a' VALUES FOR SOME ORGANOPHOSPHATE METABOLITES[a]

Compound	pK_a'
$(C_2H_5O)_2PSSH$	1.62
$(C_2H_5O)_2PSOH$	1.37
$(C_2H_5O)_2POOH$	1.49
$(CH_3O)_2PSOH$	1.18
$(CH_3O)_2POOH$	1.25
 $(CH_3O)_2P(S)S\underset{\substack{\vert \\ CH_2COOH}}{C}HOOC_2H_5$	3.0
 $(CH_3O)_2P(S)S\underset{\substack{\vert \\ CH_2COOH}}{C}HCOOH$	3.0
$(CH_3O)_2P(S)SCH_2COOH$	3.8
$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$	8.4
 *cis*-$(CH_3O)(HO)P(O)O\underset{\substack{\vert \\ CH_3}}{C}{=}CHCOOCH_3$	2.5
 *cis*-$(CH_3O)_2P(O)O\underset{\substack{\vert \\ CH_3}}{C}{=}CHCOOH$	ca. 4.2

[a] Data of Kabachnik *et al.*[50] O'Brien,[79] and Spencer.[97]

With these exceptions, it is a useful generalization that the solvent-extractable materials tend to be the more hazardous compounds, whereas the unextractable materials are usually entirely harmless. This can be convenient in differentiating between hazardous and total residues on crops or other products after insecticide treatment.

It is very common to find that a certain amount of material cannot be extracted from a homogenate. One has to decide how great a loss one can tolerate, and attempt to achieve an appropriate extraction. The difficulty varies considerably with compound and extractants. Thus, Krueger *et al.*[59] found that Co-ral was very difficult to extract with chloroform from proteinaceous solutions, a phenomenon which they attributed to protein-binding: after 1 hour only 55% was extractable from bovine plasma and 70% from milk. In both of these cases the extractability decreased with time, so that at 12 hours only 30 % was extractable from plasma, and 20 % from milk. MacDougall,[65] however, found that by extracting the tissue directly with acetone and then with benzene, Co-ral could be recovered satisfactorily; this procedure was also effective for malathion.[80] In the author's experiments, a 10 % loss of metabolites is tolerated. To achieve this, it is sometimes best to precipitate the protein from the preparation, but with rather dilute trichloroacetic acid.[58] The protein precipitate is later filtered off, mixed with Celite to make it manageable, and extracted, e.g., with chloroform and methanol alternatively. The resultant extracted materials are later repartitioned between water and solvent and added to the appropriate fractions from the initial extractions.

To illustrate these points, here is a typical procedure with malathion-treated animals:[61] 4 gm. of insects were homogenized in a Lourde's multimixer with 80 ml. of chloroform plus 80 ml. of water containing enough trichloroacetic acid to give a pH of 2.5. The mixture was filtered through Celite in a Buchner funnel, and the precipitate was washed on another filtering flask with 40 ml. each of methanol and acetone; the filtrate was evaporated to dryness and taken up in the chloroform-water filtrate, which was centrifuged and the water layer removed. The chloroform was repartitioned against water containing enough NaOH to give a pH after partitioning of 7–7.5, and the aqueous fractions were combined.

This preliminary separation of metabolites is normally necessary prior to further (usually chromatographic) separation, because most chromatographic systems are designed to resolve mixtures with a fairly limited range of polarities. Thus, no one system would resolve parathion from paraoxon, and dimethyl phosphoric acid from *O,O*-dimethyl phosphorothioate as well. This generalization may not hold where the parent is a polar compound, e.g., in an organophosphate containing a quaternary nitrogen group.

The above discussion should illustrate a vital point: some radioactivity is quite certain to be lost somewhere in the extraction and isolation procedures, and it is essential to determine how extensive this loss is. The extraction methods are inadequate if more than 10 % is lost. It can be deceptive to say that "X represents 25 % of the metabolites," for what is usually meant is that X represents 25 % of the *recovered* metabolites, and there is no guarantee that the lost fraction contains metabolites in the same ratio as in the recovered fraction. The excellence of recovery should be the first preoccupation in any metabolite study, and the % of recovery should always be specified in the results.

Preliminary studies on the efficiency of extraction can be made by adding a known amount of parent material to a preparation of the tissue, and then submitting it to the standard extraction procedure. High recoveries in such tests are no proof that high recoveries will be obtained in the actual experiments. After satisfactory extraction in the preliminary tests has been obtained, a labeled compound should be added to the material under the conditions to be used (e.g., injection into the living animal), and after allowing a typical metabolism-time to elapse, the usual extraction procedure and analyses should be carried out, and the sum of the resultant analyses should be compared with the total radioactivity applied. The total radioactivity applied may be found (*a*) by applying a known amount of material to the organism, e.g., in topical application to insects, (*b*) by measuring the uptake from a known quantity, e.g., in root uptake by plants from free solutions, and (*c*) by measuring the activity in a whole homogenate of the organism, e.g., in uptake of gaseous material by organisms.

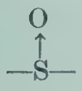
A useful device for extracting parent compounds and unhydrolyzed metabolites from fatty material (e.g., milk or whole-animal homogenates) is to bring the fat and the organophosphates into hexane, then extract several times with acetonitrile to remove the organophosphate. This device has been used by Casida's group for parathion,[1] dimethoate,[28] and Co-ral.[60] It is most effective when the organophosphate is somewhat polar.

In another special technique, of particular use in extracting hydrolysis products or when endogenous emulsifiers are present, the tissue is mixed with several times its weight of anhydrous Na_2SO_4, the resulting powder is loaded into a small column and is extracted with a solvent; carbon tetrachloride has been used for eggs, and 5% ethanol in carbon tetrachloride for liver.[76]

Separation and Identification of Solvent-Extractable Metabolites

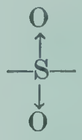
The principal techniques are paper or column chromatography, although Craig countercurrent extraction[98] has also been used.

The number of compounds to be separated is not large except in the case of alkylthioalkyl phosphorothionates, where one may have the mercapto (—S—), sulfoxide (I), and sulfone (II) derivatives of the phosphorothionate and the phosphate, a total of six compounds (if isomers are absent!).



$$-\overset{\overset{O}{\uparrow}}{S}-$$

(I)



$$-\underset{\underset{O}{\downarrow}}{\overset{\overset{O}{\uparrow}}{S}}-$$

(II)

Should one use paper or column chromatography? With paper there is less risk of losses by irreversible adsorption; the identification (usually with a spray reagent) is somewhat simpler, the preparation less, and no attention is needed while development is proceeding. On the other hand, these compounds must be run on reversed phase chromatograms in which the paper is soaked in (say) silicone, and it is not always easy to duplicate the preparation; quantitation is a little complex, since the compound may have to be eluted from the paper (densitometry—the measurement of the color density of a spot—is not very accurate but in tracer work, scanning of the paper with a counter may be quite satisfactory); sometimes the resolving distance is inadequate, i.e., it is not very practicable to work with papers of more than 1½ to 2 feet in length.

Column procedures are of more recent growth. Perhaps their major advantage is that one can extend any part of the eluting system to an unlimited extent by using gradations of mixed solvents; resolution tends to be better than with papers; the eluted compounds are immediately available for infrared or antiesterase studies; procedure can be extremely rapid, especially if there are (say) only two compounds, and one is eluted fully by a compound which does not elute the other; and the same basic system can be used analytically with (say) a six inch column, then readily extended a hundredfold if quantities are needed for (say) infrared spectra.

The present author's own rather subjective view is that papers are preferable for qualitative studies, particularly if one has little technical assistance and can afford to wait a good many hours for results. In other cases columns are preferable, unless irreversible adsorption is a problem for the compounds involved.

As for detailed techniques, the procedure for paper chromatography devised by Metcalf and March[72] is widely used. It involves treating the paper with 5% silicone in petroleum ether so that the treated paper is the apolar phase, and the eluant (upper phase of a chloroform:ethanol:water mixture 10:10:6) is the polar phase. This system is called "reversed phase chromatography," because normally the paper is the polar phase. A more recent reverse phase system[95] uses paper soaked in phenyl Cellosolve: acetone, 1:10 or 1:20, or in Cellosolve:propylene glycol:acetone, 1:1:10 or 1:1:20. The eluant is *n*-hexane:chloroform:acetonitrile, 50:5:6, or *n*-hexane:benzene:acetonitrile, 50:5:6.

A method[66] used for more polar compounds, such as schradan, involves treatment of the paper with 50% ethylene glycol in ethanol, and eluting with a vile-smelling mixture of trichlorotoluene:carbon tetrachloride:toluene, 1:1:1, saturated with ethylene glycol. Another method for polar compounds[89] uses papers treated with 20% Carbowax 300 (a polyethylene glycol material, average molecular weight 300) in acetone. The eluant is isopropyl ether containing 0.2% ammonia and 0.001% resorcinol. These last two methods are not reversed phase systems, since the paper is still the polar phase.

In reversed phase systems the less polar compounds (e.g., phosphorothionates) will stay near the origin, whereas the more polar compounds (e.g., phosphates) will move nearer the solvent front. The opposite is true for the systems in which the paper is polar.

Identification is usually made with a phosphorus-sensitive spray such as that described by Hanes and Isherwood,[41] which contains perchloric acid and ammonium molybdate, and usually requires heating or ultraviolet irradiation to develop the colors. The rapidity of color development can be used as an aid in qualitative identification: compounds such as malathion, which are rather stable to acid hydrolysis show color slowly, whereas unstable compounds such as TEPP show color rapidly. After heating (e.g., 85°C., 7 minutes) color development can be accelerated by *brief* exposure to H_2S. Fleckenstein *et al.*[32] differentiate esters which are easy to hydrolyze from those which are difficult to hydrolyze by exposing the paper after heating to H_2S and then to NH_3, at which time the blue colors from the esters which are difficult to hydrolyze will disappear.

An extremely useful spray reagent is 2,6-dibromoquinone-*N*-chloro-*p*-quinoneimine (also known as 2,6-dibromoquinone chloroimide). Menn *et al.*[69] showed that the compound is specific for sulfur-containing organophosphates, and is best when the sulfur is in the P═S form; thus a parathion isomer, containing P—S—C but no P═S, gave no color. The spray has the advantages of giving very intense and stable colors, and avoids problems connected with the acidity of the Hanes-Isherwood spray (crumbling of the paper, and corrosive properties of the spray drift). We have found that it is very unstable in acetone solution, and will last only a few days even if stored in a deepfreeze. The use of *N*-bromosuccinimide has been proposed for detecting sulfur-containing phosphorothionates.[23]

There are three types of column chromatography for chloroform-extractable compounds: silica gel, for use mainly with rather polar compounds (e.g., those 1% or more soluble in water) such as schradan,[103] dimethoate,[28] and Phosdrin;[18] and Celite or alumina for less polar materials.

Silica gel columns are prepared by adding water (or buffer if the metabolites are unstable; or acid if the materials are acidic, since it is the unionized form which is operated on) to anhydrous silicic acid, chromatographic grade. The silicic acid is best bought in bulk, as batches may vary, and it is desirable to heat it at 110°C. for 24 hours in an open dish before use, since it takes up water readily on storage. A supply can be stored in the oven at 110°C. and does not appear to deteriorate over a period of a few weeks. Some practical details are given by Bulen *et al.*[14] Elution is usually with a succession of mixed solvents of increasing polarity, e.g., hexane; hexane-chloroform 4:1; hexane chloroform 1:1; hexane chloroform 1:4; chloroform (Dauterman *et al.*[28]); the compounds are eluted in increasing order of polarity.

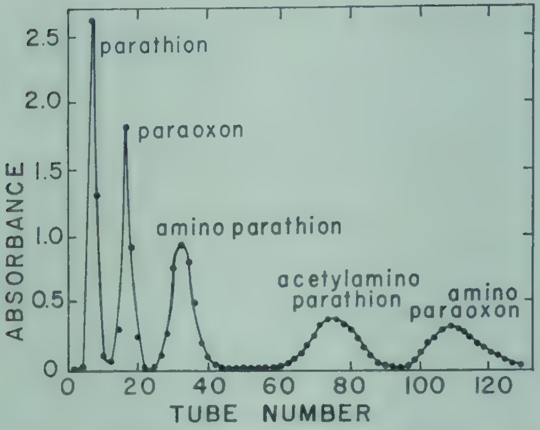


FIG. 10.1. Separation of parathion and some possible metabolites on a Celite column. From Ahmed *et al.*[1]

Celite columns have been used for malathion, acethion, Co-ral, parathion, etc. Figure 10.1 shows a separation of parathion metabolites. These columns need careful preparation, particularly with regard to uniform packing. A typical preparation: to 100 gm. of Celite add 60 ml. of methanol which is $^2/_3$ saturated with isooctane (i.e., 20 ml. methanol + 40 ml. of isooctane-saturated methanol). Mix, then slurry with about 500 ml. of isooctane which is $^4/_5$ saturated with methanol. Pack into column by layers, tamping down each layer, e.g., with a plunger made by wrapping cheesecloth around the end of a glass rod (then one can pull off the cheesecloth for easy removal of the plunger). The compounds are then added in isooctane $^4/_5$ saturated with methanol, and eluted with the same solvent.

Alumina columns are particularly useful for separating phosphorothionates from their P=O analogs, as suggested by Plapp and Casida.[86] They are easy to prepare, but it is essential to use acid-washed alumina; a highly standardized (but expensive) type is Woelm acid alumina, anionotropic, activity grade I for chromatographic analysis.* It must be kept sealed tightly after opening. The column is prepared by adding 2% or 3% by weight of water to the alumina, mixing and allowing equilibration, then slurrying in hexane, and pouring the mixture into the column. The compounds are added in hexane if phospholipids are present; the phospholipids are eluted off with hexane, then benzene is used to elute off the phosphorothionates, then chloroform is used to elute off the P=O analogs. If no phospholipids are present, the compounds can be added in benzene and the hexane-elution can be omitted.

The alumina system has the great advantage of all or none separation, i.e., when only two components are present (as is usual escept with alkylthioalkyl and related compounds) one solvent elutes one component. With an established system, collection of many fractions can therefore be omitted. It cannot be used (at any rate in the manner described above) for rather polar phosphorothionates such as dimethoate. With malathion some irreversible adsorption on the column occurs,[61] but with acethion, Diazinon, and parathion there is very little.[62] The system has been used also for ronnel, dicapthon, Chlorthion, and methyl parathion[86].

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* This is sold in the U.S.A. by Alupharm Chemicals, P.O. Box 755, New Orleans, La.

Craig countercurrent extraction has been used for separating schradan metabolites[98, 103] and Phosdrin isomers.[99] It is not recommended unless the simpler techniques of chromatography have proved inadequate, as it is somewhat cumbersome and demands fairly expensive apparatus (although its principles can be applied using multiple extractions with separatory funnels). An excellent review by Craig and Craig is available.[25]

The problem of identification disappears if good separation has been achieved, and one has pure samples of the parent compound and its oxidation product(s). In such a case, one simply determines where each appears in the chromatographic analysis, and identifies the metabolites on the basis of position.

SEPARATION OF SOLVENT-UNEXTRACTABLE METABOLITES

Once more, the main choice lies between paper and column chromatography. Basically, the arguments in favor of each are the same ones given for the solvent-extractable metabolites. Paper techniques are even simpler with the solvent-unextractable metabolites, since reverse phase procedures are not needed. However, the problem of adequate resolution is more severe than with solvent-extractable metabolites, since there are often more metabolites (e.g., 10 in the case of malathion), and some important ones are so similar as to be difficult to resolve on paper, e.g., *O,O*-diethyl phosphorodithioate and *O,O*-diethyl phosphorothioate.[81] Column procedures, therefore, offer a great advantage in that difficult areas of resolution can be extended as far as one pleases. Details of suitable column procedures have been available only since 1958 (Plapp and Casida[85]).

The classic description of paper techniques by Hanes and Isherwood, in 1949, is still the best.[41] The use of 2,6-dibromoquinone-*N*-chloro-*p*-quinoneimine, as described above, increases the sensitivity for phosphorothioates. When the pK_a values of the metabolites to be separated are known, buffered systems can be useful: thus, if an acid A has a pK_a of 2, and B has a pK_a of 4, in a system at pH 3, A will be ionized and have a small R_f, whereas B will be un-ionized and have a large R_f.[81] One would perhaps anticipate streaking under such conditions, but such is not the case. The R_f values of twenty organophosphate metabolites in two solvent systems have been established.[85]

The development of ion-exchange columns for analysis has been most important. The following description is based mainly on Plapp and Casida.[85] The principle is that if one adds a mixture of acids to an ion-exchange column and then elutes it with progressively more acid solvents, each compound will remain on as long as it is ionized, i.e., when the pH is above its pK_a, and be removed when un-ionized, i.e., when the pH is below its pK_a. As the more acidic compounds are also rather tenacious, methanol is added progressively to strip these off. In fact, the columns depart rather far from obedience to this pK rule, and must be considered as acting concurrently as adsorption columns as well as ion-exchange columns. Figure 10.2 shows the results from an actual experiment on dimethoate metabolism.

The technique uses a series of pairs of eluants, successive ones being more acid and having increasing concentrations of acetone or methanol, which elute off the more "difficult" compounds. Usually, every successive investigator has made small improvements in the technique, particularly with respect to the eluant pairs, and even so there is undoubtedly room for further improvement. Fortunately, the phosphorus-containing metabolites are far less diverse than their parent compounds, and the commonest are only of four types: $(RO)_2POOH$, $(RO)_2PSOH$, $(RO)_2PSSH$, and H_3PO_4.

In the experiment of Fig. 10.2, 500 ml. of each of the eluant pairs were used. The solvents are added to the column in such a way that the composition of the eluant is constantly changing. To achieve this, one may use a very simple mixing device as in Fig. 10.3A, which is air-stirred and to which pressure cannot be applied (these columns take many hours to run, and pressure is often needed to maintain a good flow rate). Another

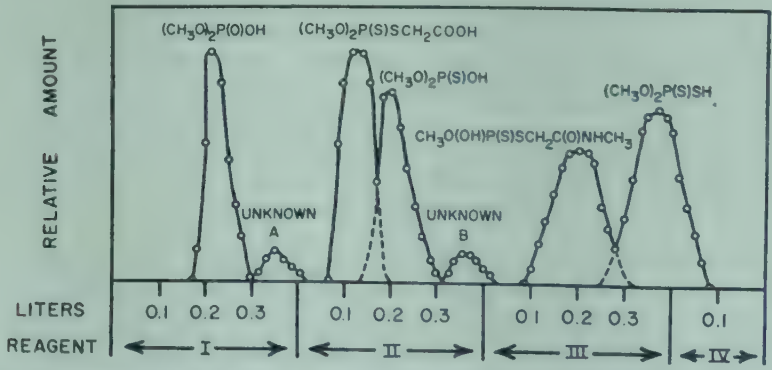

Fig. 10.2. Separation of the ionic metabolites of dimethoate on an ion-exchange column (Dowex 1). From Dauterman *et al.*[28]

Eluant pairs:

I, HCl pH 2/HCl pH 1

II, HCl pH 1 + methanol (1:3)/N HCl + methanol (1:3)

III, N HCl + acetone (1:3)/conc. HCl + water + acetone (1:1:6)

IV, Conc. HCl + water + acetone (1:1:6).

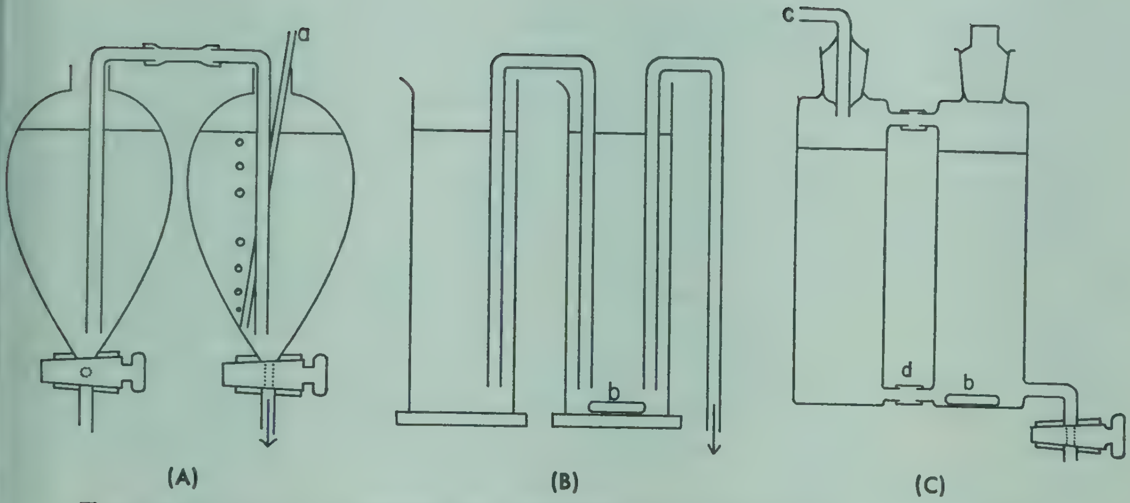

FIG. 10.3. (*A*, *B*, *C*) Devices for delivering mixed eluants in gradient elution.
a: air inlet for stirring;
b: stirrer magnet for electric stirring;
c: air inlet for elution under pressure;
d: Some form of clamp or stopcock should be added here.

simple device, Fig. 10.3B, uses graduate cylinders and can be used either with a magnetic stirrer or an air stirrer. It is perhaps a little cumbersome to suspend over the column, for gravity-feed is the usual procedure; however, to avoid this cumbersome aspect and to assure a known and adjustable flow rate, Bock[11] uses a small accurate pump to deliver from the cylinder to the chromatograph column.

The apparatus we have found best is shown in Fig. 10.3C. It is magnetically stirred, can be run under pressure, and is free of the siphon problem which is a considerable nuisance with the other devices. The shape and dimensions of the two vessels is important, and by varying them one may vary the rate of change of pH in the mixed eluants, as discussed by Bock and Ling.[12] In general, if one compound only is eluted by a given eluant pair, the way the pH changes is not critical, but with compounds not easily resolved a slow pH change during their elution improves separation.

It is desirable to include known synthetic nonradioactive metabolites in each run, then to assay each fraction collected (10 ml. is a convenient volume) for phosphorus and for radioactivity. Identification is then reliably given by coincidence of peaks of the known and unknown. The phosphorus assay can be quick, approximate, and automatized; an accuracy of 10% is ample. The method of Rockstein and Herron[92] is simple and avoids the obnoxious odor of the amidol-sodium bisulphite used in the otherwise excellent method of Allen.[2]

The fraction collector used in these and other chromatographic columns should utilize a volume-measuring device, the simplest being a Soxhletlike siphon. The standard fraction collectors sold in the U.S.A. are rather expensive, costing at least $500. A much cheaper and equally efficient collector can be obtained in England, costing about $200 in 1954.*

The radioactivity of the eluted fractions can be counted with an end-window counter, the samples being counted wet (i.e., 1 ml. of sample placed on a planchette, greased at the rim to prevent creeping) or dried first in the planchette, using a fan to speed the process. Greater sensitivity with less risk of losing activity by volatilization can be achieved by using a liquid counter; this has the disadvantage of needing more material (commonly 10 ml.), requiring washing between samples, and being unsuitable for some automatic counting and recording equipment. Liquid counting is most useful for prefractionation studies, e.g., of activity in whole homogenates, blood, urine, or milk.

Spectroscopy

There are three kinds of spectroscopy of use to workers with organophosphates: ultraviolet (UV), infrared (IR), and nuclear magnetic resonance (NMR). The first is probably available to 95% of laboratories, the second to about 10%, and the third to about 0.01%.

ULTRAVIOLET SPECTROSCOPY

Ultraviolet absorption is usually weak or absent (i.e., absent from the readily measured part of the ultraviolet spectrum) unless a double bond is present. The presence of a system of conjugated double bonds (i.e., alternating single and double, such as —C═C—C═C—) intensifies absorption and also shifts the absorption to a longer wavelength; such a shift is termed the bathochromic effect. The most usual conjugated system is that of aromatic compounds, and the most interesting organophosphates in this connection are therefore those with aromatic substituents, e.g., parathion, Co-ral, Guthion.

Ultraviolet data have been used by Gersmann and Ketelaar[37] to determine what contribution "opposing resonance" makes to the high free energy of hydrolysis of some aromatic phosphate esters.† They found that, in fact, opposing resonance was a minor factor. In three *p*-nitrophenyl phosphates, including paraoxon, the absorption maximum was less (from 2 to 6 mμ) for the phosphate than for the corresponding phosphorothionate. The hypsochromic effect (i.e., shifting of absorption to a shorter wavelength) upon the absorption of the *p*-nitrophenol group was, therefore, greater with a phosphate than a phosphorothionate. This was attributed to the greater electronegativity of ═O than ═S, resulting in better stabilization of one of the resonance forms.

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* From A. Gallenkamp and Co., 17 Sun Street, London E.C.2.

† "Opposing resonance" is the term used by Oesper[82] for a phenomenon that can occur if an ester or anhydride AB hydrolyzes to give A + B, and when A and/or B have more resonance forms when separated than they had in the condensed state. In such a case, hydrolysis is favored (i.e., the free energy of hydrolysis is increased) because of the extra stability of A and B in their separated as compared to their condensed state.

Data on nonaromatic compounds are rather sparse. Spencer *et al.*[99] have used the absorption of Phosdrin at 218 mμ as an assay of fractions from the Craig countercurrent separation. Serra and Malatesta[94] report maxima in alcohol at 261 and 256 mμ for diisopropyl phosphorochloridate and diisopropyl phosphorochloridite, respectively.

Pianka[84] observed that phosphates have a characteristic absorption maximum in hexane at 214–219 mμ, presumably due to P═O. His study on the effect of substituents revealed that (*a*) replacement of an ethoxy by a dimethylamino group had little effect in contrast to the bathochromic effect of this replacement in conjugated systems; (*b*) replacement of an ethoxy or of a dimethylamino group by chlorine narrowed the absorption band, with no change in maximum. Replacement by fluorine, however, strongly decreased absorption.

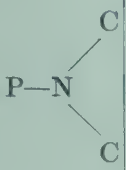
In summary, ultraviolet spectroscopy has fairly limited use as an assay procedure, and little usefulness for identification other than in distinguishing the presence or absence of conjugated systems.

INFRARED SPECTROSCOPY

Infrared (IR) spectroscopy is an invaluable tool for identification, for determinations of purity, and for establishing the structure of unknown compounds. For identification and determination of purity, one simply traces (preferably on the same chart) the IR spectrum of a sample of a known pure specimen, and precise overlap indicates identity. However, this method will only indicate an impurity present in small percentage if that impurity has a strong absorption in a region where the pure material has but a weak absorption.

The important characteristic of absorption in the infrared is that every bond in the molecule has a characteristic absorption maximum, which is usually modified to a fairly small extent by the nature of its neighboring substituents. Consequently, one can draw up tables showing the expected range in which the absorption maximum for a given bond may fall. Table 10.2 lists some of the absorptions likely to be of interest to those who work with toxic phosphorus esters. A given bond may have more than one absorption maximum for various reasons. First, bonds can vibrate in different ways, and each way has its own frequency of vibration and hence

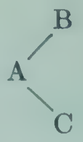
TABLE 10.2. INFRARED ABSORPTION MAXIMA FOR ORGANOPHOSPHATES[a]

Group	Frequency	Reference	Group	Frequency	Reference
P—H	2350–2440	26	P—O—C (aromatic)	1190–1240	8
P—F	850–980	26			
P—Cl	430–585	26	P—O—P	940–970	8
P═O	1170–1310	8, 26, 42		714	45
P═S	700–770	26	P—NH—	3300–3500	8
	600–750	8			
	816	42			
P—C (aliphatic)	650–750	26	P—N in 	1000	45
P—phenyl	ca. 1000 and ca. 1440	26			
	1435–1450	8	CH_2	2817–3008 and 1368–1400	45
P—O—H	2550–2700	8, 26			
P—O—C (aliphatic)	1030–1090	8, 26		2926 and 2853	8
			CH_3	1440–1488	48
P—O—methyl	1190	8		2962 and 2872	8
P—O—ethyl	1160	8, 26			

[a] Data are as frequency in cm.$^{-1}$ *Note:* If frequency (cm.$^{-1}$) = F, then wavelength (mμ) = 10,000/F. Data of Daasch and Smith,[26] Bellamy,[8] Henglein *et al.*,[42] and Holmstedt and Larsson.[45] See also Corbridge and Lowe,[24] Maarsen *et al.*[63, 64]

its own absorption. For example, in A—B, A and B may move in and out like balls on a coil spring (this results in a "stretching frequency") or may vibrate as if they were attached to opposite ends of a vibrating reed (resulting in a "bending" or "deformation frequency"). Secondly, a given fundamental frequency has a number of overtones (or harmonics); these will be seen only if the fundamental frequency is of high intensity. These overtones are similar to those familiar in the case of sound waves, but they are not often at precisely double the basic frequency. The first overtone can be useful, e.g., in the case of C—H stretching, for diagnosis using the near infrared region. Finally, two frequencies can interact to give an absorption corresponding to a frequency which can be the sum or the difference of the basic frequencies.

Besides frequencies caused by single bond such as A—B, one also may have group frequencies; thus



may have a frequency caused by a vibration of A—B with respect to A—C, as the angle between them opens and closes. Different geometrical isomers often have IR spectra which differ in some important way. *cis*-Phosdrin, for instance, has an absorption at 897 cm.$^{-1}$ lacking in the *trans* form, which has an absorption at 913 cm.$^{-1}$ instead.[99]

Infrared spectra can be run on gases, liquids, or solids, but solutions in organic solvents are most often used. The solvent itself can interfere in two ways. All solvents have some regions of strong IR absorption, and as a result the absorption of the compound under study will be obscured in these regions. For a complete spectrum it is, therefore, necessary to run two spectra in two solvents selected so that any part of the infrared region can be seen in one or the other. For this purpose, carbon tetrachloride and carbon disulfide are an excellent pair. However, most workers instead select one solvent which blanks out regions of small interest. Chloroform is a popular solvent in this regard, its virtue lying more in its excellence as a general solvent than in its IR transparency. Perhaps the best common solvent for transparency is carbon tetrachloride, but its solvent power is not spectacular. The whole question of solvent selection is dealt with admirably by Jones and Lauzon.[49]

The second interference caused by solvent occurs if there is bonding between solute and solvent. On page 52, the use of this effect in determining the extent of hydrogen bonding has been described. Interactions between solvents and organophosphates and their effects upon absorption are described by Geddes.[36]

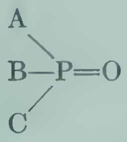
Water is for most IR purposes of no use as a solvent: it has too much absorption in the IR region and damages the rock-salt cells normally used for containing the sample. Solutions in organic solvents must, therefore, be scrupulously dried before submitting them for IR analysis. However, by using a special silver chloride cell, aqueous solutions can be studied. Fortunately, D_2O is transparent where water absorbs, and vice versa, so that by running spectra in both liquids, the IR range can be covered. This procedure is discussed by Blout.[10]

In most cases, however, water must be avoided. If a material for analysis is insoluble in organic solvents, it can be formulated by the use of a special press as a solid in a pellet of potassium halide, usually KBr, which is transparent in the IR region.

While discussing these practical points, it should be mentioned that relatively large quantities of material are needed for IR analysis, in contrast to UV analysis. Normally, about 0.06 ml. of a 10% solution are needed, thus using 6 mg. of material. However, with special microattachments, a few micrograms can be used.

A knowledge of the factors which cause shifts in the absorption maxima is useful in the correct assignation of observed maxima to particular bonds. It can also be used to assist in identification of neighboring substituents.

Daasch and Smith[26] pointed out in 1951 that the P=O stretching frequency was increased by substituents on the phosphorus which were electronegative (and thus electrophilic*). This observation was greatly expanded by Bell *et al.*[7] in 1954. They studied an impressive list of 59 compounds of the type



where A, B, and C were halide, alkoxy, phenoxy, etc. For each substituent they obtained by computation a parameter which they called the "phosphoryl absorption shift contant," which was essentially equivalent to a Pauling electronegativity constant. For any given compound they then summed the shift constants for A, B, and C. The sums for the compounds were then plotted against the observed P=O frequency for the compounds. The results are given in Fig. 10.4. This fine study shows clearly that electronegativity is by far the most important characteristic of neighboring groups in determining their influence upon the frequency of P=O. Another extensive study on P=O stretching frequencies in 30 compounds, many of them of biochemical and toxicological interest, was made in 1959 by Ketelaar and Gersmann[53] who again showed the overriding importance of the electronegativity of substituents on the phosphorus. Similar conclusions follow from the study described on page 90 on the effect of substitutions in determining P—O—C frequency in aromatic phosphates, and from work on O—H in phenols,[47] and on C—O and C=O— in carboxyesters.[67]

Clearly, the above considerations can be applied in the identification of partially unknown phosphorus compounds: if one of the substituents on the phosphorus were unknown, its electronegativity could be calculated. Unfortunately, this approach can be used only for an absorption whose response to neighboring groups has been established, since the direction of the response varies. For instance, the studies cited above show that electronegative substituents increase the frequencies of P—O—C in aromatic esters, of C=O and C—O in carboxyesters, and of P=O in phosphoryl compounds, but decrease the frequencies of O—H in phenols and catechols (in this case for both the intramolecularly hydrogen-bonded and free forms of hydroxyl).

The cause of the modification of the P=O frequency has been attributed by Hooge and Christen[46] to a modification of the degree of ionic character of the bond. The bond has two principal resonance forms:

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* Electronegativity is a direct measure of the electrophilic nature of an element or group, since electronegativity measures the power of attraction for electrons in a covalent bond.[83]

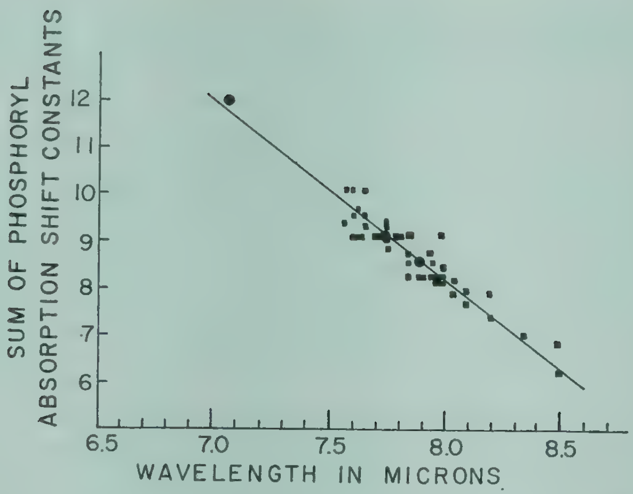


FIG. 10.4. Relationship between the wavelength of infrared absorption for P═O and the electronegativity of substituents, in pentavalent organophosphates. From Bell *et al.*[7] ●: Values for $F_3P(O)$, $Cl_3P(O)$, and $Br_3P(O)$ on which the values for the shift constants were based.

$X_3P{=}O$	$X_3P^+{-}O^-$
Double bond form	Ionic form

In the absence of electrophilic substituents, the bond is 80% ionic. Electronegative substituents, by drawing electrons from the phosphorus, increase the double bond character and hence the P═O frequency. The P═S bond, on the other hand, is only 8% ionic, and so is far less capable of modification. Consequently, the electronegativity of substituents has little importance in determining P═S frequency. Some other rather complicated factors become important, whose over-all effect may be summarized as follows, where F_{PX} is defined as the frequency of the isolated P—X bond and F_{PS} as the frequency of the unmodified P═S bond: (*a*) if F_{PS} is very close to F_{PX}, the P═S bond in $X_3P{=}S$ does not have an absorption of its own; (*b*) if F_{PS} is greater than F_{PX}, then the P═S absorption of $X_3P{=}S$ is greater than F_{PS}; (*c*) if F_{PS} is less than F_{PX}, then the P═S absorption of $X_3P{=}S$ is less than F_{PS}.

NUCLEAR MAGNETIC RESONANCE

The most recent addition to the techniques for elucidating molecular structures is NMR or nuclear magnetic resonance, first used in 1946, and applied to phosphorus by Knight[54] in 1949. It utilizes the principle that nuclei of some isotopes behave like spinning magnets, which when exposed to a particular magnetic field and a particular electromagnetic wavelength (in the radio-frequency range) can absorb energy. This absorption is a resonance phenomenon, not unlike that familiar with sound waves, when an object may resonate when exposed to a specific audio-frequency. To measure the NMR absorption, the most usual arrangement is to place the sample between two very large electromagnets, expose it to a fixed radio-frequency from a transmitter, and have a radio-frequency detector so arranged as to respond when resonance occurs. The very large magnetic field is then varied within a very small predetermined range of a few milligauss, and the response from the detector is observed with a cathode-ray oscilloscope. Peaks are then observed to occur at characteristic field strengths, and the area under the peak is proportional to the concentration of the isotope.

Only a few isotopes have useful nuclear magnetic resonance, the most important (in order) being H^1, F^{19}, P^{31}, O^{17}, and C^{13}. Since naturally-occurring phosphorus is about 100% P^{31}, it is a very convenient element for measurement.

Fortunately, the absorption peak of an isotope is dependent upon its electronic environment, which in turn is a function of the substituents attached to it. The precise position of the peak, therefore, can be used to determine what groups are attached to that atom, and what sort of bonds are involved. For example, the phosphorus in $(RO)_2P(S)X$ has a different peak from the phosphorus in $(RO)_2P(O)X$. Thus, a symmetrical pyrophosphate such as $(RO)_2P(O)OP(O)(OR_2)$ shows only one peak for phosphorus, but $(RO)_2P(S)OP(O)(OR)_2$ would have two peaks, each having half the area under them of that for the symmetrical compound (assuming equimolar concentrations of the compounds).

The shift in the peak caused by any particular set of substituents is called the "chemical shift" (δ). An arbitrary reference point is needed from which to calculate chemical shifts, and for phosphorus the position of the P in H_3PO_4 is usually used. The chemical shift is defined as a fraction of the applied field:

$$\delta = \frac{Hs - Hr}{Hr} \times 10^6 \text{ p.p.m.}$$

where Hs and Hr are the field-strengths for the maxima of the sample and of the reference (H_3PO_4) respectively. The chemical shift δ is dimensionless.

The other useful diagnostic effect is called "spin-spin coupling." It is observed that with high resolution spectroscopy, the expected peaks found on the basis of the previous discussion are often split into a series of subpeaks. Consider, for instance, a phosphorus atom attached to a hydrogen atom, which has itself a pronounced NMR peak, but in a different range from that of phosphorus. The resonances of the phosphorus and hydrogen are not completely independent, and interaction occurs. Now the hydrogen nucleus can spin in either of two directions, and normally half are spinning one way, half the other. Since each type interferes in a different way, there are two interaction peaks, and the expected single phosphorus peak is split into two subpeaks, one shifted by $+x$ from the noninteraction position, the other by $-x$.

Spin-spin coupling can also occur between two similar isotopes (e.g., two phosphorus atoms) but only if they are not identical in their environment. Thus, there could be no coupling in a symmetrical pyrophosphate, but there could be in an anhydride such as $(CH_3O)_2P(O)OP(S)(OCH_3)_2$. However, the extent of spin-spin coupling decreases rapidly with an increasing number of atoms interposed between the interacting nuclei.

One can readily distinguish spin-spin splitting from a chemical shift effect by performing experiments at two magnetic field strengths: spin-spin separation then has the same absolute value in both cases, whereas the absolute value of the chemical shift is dependent upon the intensity of the applied field.

Let us now consider some practical problems which have been solved by NMR. Muller and Goldenson[74] used it for studying the rate of Systox isomerization. The chemical shift caused by P(S)O was amply different from that caused by P(O)S, so that in the isomerization mixture two peaks were found, one for each isomer, and the areas under them measured the concentrations of each. With this technique they found that the rate was intermediate between first- and second-order, being represented by an equation of the form:

$$-\frac{dc}{dt} = \frac{k_1 c}{k_2 + c}$$

This explanation helps to resolve the different findings of other workers using different techniques (see page 56).

The chemical shifts caused by substitutions have been exhaustively studied by Van Wazer *et al.*,[105] Finegold,[31] and Muller *et al.*[75] The effects found differed profoundly with the number of atoms bound to the phosphorus. With derivatives of phosphine, PH_3, values for eleven substituents were reported, such that the total shift for any compound could be calculated by summing the individual values. In quadruply bond compounds, such as phosphates and phosphonates (in which the phosphorus, although pentavalent, has four groups bound to it) the effect of substituents was less regular. Table 10.3 shows that the effect of sulfur was particularly dependent upon the type of compound substituted. This is unfortunate, as it is these compounds which are of main interest toxicologically. However, within many types, there was good similarity in chemical shift. Thus, six dialkyl alkylphosphonates had δ values between -30 and -32.5. Four *O,O*-diethyl-*S*-alkyl phosphorothiolates had δ values between -26.4 and -26.6. In general, it should be fairly easy to distinguish between isomers such as P(S)O and P(O)S, and between parent and oxidation products such

TABLE 10.3. CHEMICAL SHIFTS PRODUCED IN QUADRUPLY BOUND PHOSPHORUS BY SUBSTITUTING P—O BY P—X[a]

X	Number of types examined	Range of $-\delta$ (p.p.m. per substitution)
H	5	2–8
C (aliphatic)	3	22–37
C (aromatic)	7	6–23
N	9	3–11
F	1	−10
P	1	19
S	8	25–71
Cl	5	0–10

[a] From Van Wazer *et al.*[105]

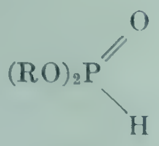
as P(S)O and P(O)O. In fact, within any one type of compound, any modification in the atoms bound directly to the phosphorus should be easily demonstrable. It is in such modifications that NMR can be most useful.

Considerable light has been shed upon the constitution of possible tautomeric mixtures by use of the chemical shift. It has been shown[31] that $(C_2H_5O)_2POSH$ is mainly in the P(O)SH form, whereas the related phosphonates, $CH_3(C_2H_5O)POSH$ and $C_2H_5(C_2H_5O)POSH$, are mainly in the P(S)OH form. However, the salt forms of these compounds are not necessarily analogous to their parent acids: thus, $(C_2H_5O)_2POSNa$ is mainly in the P(S)ONa form. However, in the above phosphonates, the sodium salt retains the parental structure.

The spin-spin coupling effect has been utilized by Callis *et al.*,[15] but not for compounds of direct toxicological importance. An example of a simple yet valuable elucidation, is that of the dialkyl phosphites, for which two possible structures have been proposed:

$(RO)_2P\text{—}OH$

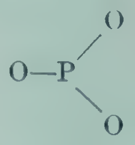
(I)



$(RO)_2P(=O)H$

(II)

From the fact that considerable spin-spin splitting of the P resonance by the H was observed, H must be attached directly to P. Furthermore, no peak was found at the chemical shift region for compounds containing:



Consequently, (II) is the predominating species (95% or more). A danger in interpretation of spin-spin splitting, particularly without supporting data, is that weak splitting can be induced by atoms not directly attached to the atom whose NMR is under study.

Finegold[31] has given a very useful discussion on the complementary uses of IR and NMR, indicating the kinds of problems most suited to each. An excellent brief account of NMR is given by Roberts.[91]

Refractive Index

One of the easiest parameters to obtain for liquid organophosphates is the refractive index. Recent work by Sayre[93] has shown how this may be used to establish the identity of unknown phosphorus compounds and check the corrections of synthesized known compounds. In fact, Sayre used his predicted values to question the accuracy of published experimental values, and it was found that indeed a clerical error had been made in reporting them!

The procedure is simple. The refractive index at 20°C., n_D^{20}, is predicted by dividing the sum (S) of the bond refraction constants for every bond in the molecule by the molecular weight (M)

$$n_D^{20} = S/M$$

These bond refraction constants are listed in the tables of Sayre[93] for phosphorus bonds, and in the tables of Vogel[109] for carbon, hydrogen, and nitrogen bonds. Some of the more generally useful constants are listed in Table 10.4. If the observed values are not measured at 20°C., they should

TABLE 10.4. BOND REFRACTION CONSTANTS[a]

Bond	Constant	Bond	Constant
P—C	25.57	C—H	3.87
P—Cl	68.57	C—C	12.86
P—F	34.98	C=C	9.39
P—H	16.16	C—O	17.71
P—N	29.28	C=O	29.39
P—O	28.11	C—Cl	56.80
P=O	22.17	C—Br	124.51
P=S	54.26	C—S	32.84
P—S	47.07	C—N	14.51
N—H	7.26	O—H (alcoholic)	13.15
N=O	32.26	O—H (acidic)	10.54
		S—O	37.1
		S→O	20.84

[a] Data of Sayre[93] and Vogel *et al.*[109]

be corrected by subtracting 0.0004 per °C. in excess of 20°C. (or adding this amount if the determination was at less than 20°C.). Thus, for trimethyl phosphate one adds 3 × 25.57 for three P—C bonds, 9 × 3.87 for nine C—H bonds, and 22.17 for one P═O bond. The sum (133.71) divided by the molecular weight (109) gives a predicted refractive index of 1.2267. The observed value (given in the *Handbook of Chemistry and Physics*)[44] is 1.220 at 15°C. which when corrected to 20°C. gives 1.222. The prediction is thus accurate to 0.5%. In general, one may expect an accuracy of the order of 1%.

Refraction constants have been used by Gillis *et al.*[38] to show that, in phosphates, the phosphoryl oxygen is bound by co-ordinate rather than double bonds to the phosphorus, i.e., P→O rather than P═O.

Esterase Determinations

There are numerous ways of determining esterases, all based on one of two principles. One may determine the acid production when the ester substrate is hydrolyzed, and this can be done manometrically, by following the pH change in a mildly buffered system (with a pH meter or by following the color change of an indicator), or by titrating back to the original pH. Secondly, one can measure spectrophotometrically the disappearance of substrate or the appearance of products.

MANOMETRIC TECHNIQUES

These have been exhaustively reviewed by Umbreit *et al.*,[104] Dixon,[29] and Augustinsson.[6] Only minor points need to be added.

A popular bicarbonate buffer for cholinesterase assay is the "R_{30}" buffer of Augustinsson, which contains Na^+, K^+, and Ca^{++}. However, it is now known as the result of the extensive experiments of Smallman and Wolfe[96] and Chadwick *et al.*[19] that the activating effects of salts upon cholinesterase is quite nonspecific. It is therefore unnecessary to use several salts in the buffer. An appropriate level of NaCl is all that is required.

When the side-arm contents are tipped into the main compartment, various changes can occur which give rise to gas evolution or uptake: if solvents (such as ethanol) are present in one compartment, volume shrinkage may occur on mixing, and the solubility of CO_2 in the system may be reduced by the solvent. Again, unless all components are in bicarbonate, one may have some acid material, particularly in the substrate solution, which will cause a CO_2 evolution on mixing. In view of these possibilities, it is a good device to mix the flask contents before closing the manometers. Time should then be left for temperature re-equilibration (e.g., 5 minutes) prior to closing the manometers.

It is, for some experiments, an undesirable feature of many standard procedures that the system is exposed to a high pH for the period between addition of sodium bicarbonate to the flasks and the time of gassing. This can be avoided by having a buffer such as phosphate or tris(hydroxymethyl)aminomethane in the system. In one particular experiment, by using this technique, the pH of the system at pH 7.0 was only increased to pH 7.3 by bicarbonate, and then fell to pH 7.2 after equilibration with 5% CO_2 in N_2.[78] The presence of the additional buffer has an apparent inhibitory effect upon enzymic activity, due to the phenomenon of CO_2 retention, the apparent inhibition being commonly of the range 20–30%; its extent is related directly to the concentration of the additional buffer.[9]

It is not always realized that the pH changes during the course of a manometric assay. Birmingham and Elliott[9] calculate that in an experiment in which 20 micromoles of acid were produced, (i.e., a CO_2 production of 448 μl.), the pH could drop from 7.28 to 6.75.

Standard manometric assay is unsuitable for very small enzyme concentrations, but can be modified by decreasing the flask volume and the density of the manometer fluid. It cannot be used for very low substrate concentrations, for which a method is necessary that permits measurements immediately after addition of substrate. It is also of limited use for the study of variation of activity with pH, since one is restricted to the effective buffering range of bicarbonate. Since the pK' for bicarbonate is 6.4,[44] the buffering range is about 5.5 to 8.0.

POTENTIOMETRIC METHODS

Tammelin *et al.*[101, 102] has given a number of experimental details. These include the so-called "delta-pH method," in which the pH is allowed to change, and the extent of the change is used as a measure of the enzyme activity; and the "pH-stat method" in which the pH is kept constant by alkali addition, whose volume measures enzyme activity.

The "delta-pH method" was used in 1949 by Michel,[73] and was later modified for use as a method for determining organophosphate residues.[37, 77] It is extremely simple, uses a minimum of apparatus, and many samples can be assayed in a day. Its drawbacks are (*a*) fairly low accuracy, particularly as the pH change may be well under 1 unit, and most pH meters measure to only 0.02 units, (*b*) esterases vary in activity with pH, so the reaction rate changes throughout the reaction, (*c*) interference is produced by any material with buffering action, and (*d*) for those substrates with significant nonenzymic hydrolysis rates, it is not always easy to find the appropriate correction, because one must, for the enzyme-free control, use some material whose buffering action is precisely like that of the enzyme source. If the source is crude (e.g., a whole housefly homogenate) it may have a strong wide-range buffering action which has to be mimicked by some appropriate protein solution. In some cases, it may be easier to use, for the enzyme-free control, the standard source treated with an excess of an inhibitor.

The pH-stat method was used for cholinesterase by Glick in 1938,[39] and has been reviewed by Jacobsen *et al.*[48] It is valuable for its accuracy and sensitivity. Its special feature is that one may study, with ease, a reaction at a series of pH's, including those that lie outside the bicarbonate-buffer range (which is the only range accessible to the manometric technique). Also, the undesirable pH variation of the delta-pH method is avoided. It is better than the manometric method for measuring activities at low substrate concentrations, for the reaction under such conditions may be over in 5 minutes, due to the substrate being consumed, and only in the pH-stat method may one study the reaction immediately after addition of the substrate. Another advantage it has over manometric or delta-pH techniques is that there is no interference (except perhaps in sensitivity) by buffering materials. Its drawbacks are that it is relatively slow, and that one has all the mechanical and analytical problems associated with titrimetric techniques.

SPECTROPHOTOMETRIC METHODS

The best known colorimetric method for cholinesterase depends on the determination of the acetylcholine left at the end of the reaction, using the method of Hestrin.[43] Its use for cholinesterase assay has been described by Metcalf,[70] Robbins *et al.*,[90] Fleisher *et al.*,[33] and Vincent and Segonzac.[108] It has the advantage of extreme sensitivity, and is best for measuring very small quantities of enzyme, e.g., for microassays to detect organophosphate poisoning, or to study the small residual levels in heavily poisoned animals. It is rather susceptible to interference by cholinesterase inhibitors, and is, therefore, not particularly well-suited for *in vitro* inhibition experiments.

The color change produced by the acid produced in a feebly buffered phenol red solution has been used by Gregoire *et al.*,[40, 40a] who measured absorption at 558 mμ.

The turbidity caused by the production of acid in a casein solution was used by Polonovski *et al.*[87] to follow acetylcholine hydrolysis, using a photometer with a red filter.

Several authors have employed unusual substrates whose hydrolysis is accompanied by a change in color or in ultraviolet absorption, e.g., the change in ultraviolet absorption at 240 mμ of benzoylcholine,[52] at 229 mμ of acetylthiocholine,[34, 100] or at 250 mμ of butyrylthiocholine.[100] In the visible range, the color changes at 625 mμ for indophenylacetate,[4] or at 600 mμ for a mixture of indophenol and acetylthiocholine[34] have been used.

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APPENDIX 1

Electronic Interpretations*

The Underlying Concepts

Consider any covalent molecule containing more than one element; it is found that the valency electrons are not evenly distributed, for some elements have a greater affinity for electrons than others, and consequently draw to them some of the electrons which belong to their neighbors. We have the molecule A → B if B has a greater affinity for electrons than A. The arrow here implies a shift of electrons to B. In fact, if A and B are different, we can only have A → B or A ← B, for the electron affinities of A and B must differ. Whole groups also differ in electron affinities, for instance, $—NO_2$ can draw electrons away from $—CH_3$. An atom or group which can draw electrons to it is said to be electron attracting or electrophilic, as we shall (incorrectly but conveniently) call it. The reference point is the hydrogen in a hydrocarbon chain: electrophilic groups are defined as those which are better than hydrogen at drawing electrons from (for instance) $—CH_3$. A group which is less electron attracting than hydrogen is said to be electron repelling, or nucleophilic as we shall call it. The terms electrophilic and nucleophilic are more correctly used to describe the properties of reagents rather than substituents, but there are certain advantages to our incorrect usage which will be commented on below. We shall use the terms both for reagents and for substituent groups.

There are two important reasons for studying electronic effects. They influence properties such as polarity, acidity, and basicity, and they influence rates and directions of reactions.

There are five different electronic effects: inductive, field, mesomeric, inductomeric, and electromeric. We shall discuss only the first three which are of importance in influencing polarity, acidity, and basicity. These three and also the inductomeric and electromeric effects are of importance in reaction rates; but the latter two effects are rather complex, and have seldom been used in interpreting reaction rates of organophosphates.

It is important to note that one cannot say "chlorine is an electrophilic substituent." The electronic effect must also be specified; for instance, many groups have an electrophilic inductive effect but a nucleophilic mesomeric effect. If, by an oversight, one does say "X is electrophilic," the implication usually is that one is discussing the inductive effect.

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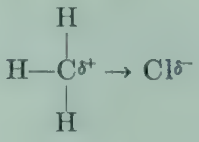
* The approach and terminology used here is based on the extensive text of C. K. Ingold ("Structure and Mechanism in Organic Chemistry." Bell, London, 1953) and the short but extremely clear book of J. W. Baker ("Electronic Theories of Organic Chemistry." Oxford Univ. Press, London and New York, 1958).

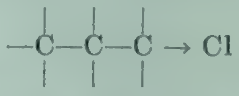
The Inductive Effect

This is the simplest and most important effect. We can arrange certain groups in order of the increasing tendency to draw electrons from neighboring atoms and, consequently, to create local changes in electron density. These local changes are indicated by the signs δ^- for an increase in electron density and δ^+ for a decrease. The inductive effect is this simple attraction of electrons along a bond. It is symbolized by placing an arrowhead on the bond indicating the direction of electron movement. For instance, Cl has an electrophilic inductive effect so that we may represent methyl chloride as:



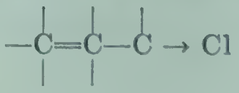
$$H-\overset{\displaystyle H}{\underset{\displaystyle H}{C^{\delta+}}} \rightarrow Cl^{\delta-}$$

The δ signs represent partially charged states known as "formal charges." The inductive effect can be transmitted along a chain of atoms, but becomes rapidly weakened in the process. Thus, in



$$-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}} \rightarrow Cl$$

the furthest carbon is influenced very little by the chlorine. However, a double bond transmits better than a single, so that the furthest carbon in



$$-\overset{|}{\underset{|}{C}}=\overset{|}{\underset{|}{C}}-\overset{|}{C} \rightarrow Cl$$

is subject to some influence.

Now, we must consider what groups are inductively electrophilic and why; and what importance this effect has. The simplest effects are with ions: negatively charged groups naturally repel electrons, positively charged groups attract them. Hence $-COO^-$ is strongly nucleophilic,

$$-\overset{+}{N}(CH_3)_3$$

is strongly electrophilic. In this connection it is important to note that the $-NO_2$ group has the form:

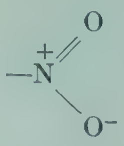


TABLE A1.1 INDUCTIVE EFFECTS[a]

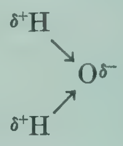
Inductive effect	Reaction
Nucleophilic:	$—O^- > S^-$
	$—C(CH_3) > CH(CH_3)_2 > CH_2CH_3 > CH_3$
Electrophilic:	$—F > —Cl > —Br > —I$
	$=O > =S > OH > OS$
	$—OR > SR$
	$F > OH > NH_2 > CH_3$
	$—C{\equiv}C— > —C{=}C—$
	$—\overset{+}{N}R_3 > —NO_2 > —NR_2$

[a] *Note:* A more correct usage is to call the nucleophilic inductive effect "the +I effect" and the electrophilic inductive effect "the −I effect." Unhappily, some authors use precisely the reverse nomenclature; hence the above usage.

the $\overset{+}{N}$ is nearer to the point of attachment than the O^-, and the over-all effect is therefore as a cation. The reasons why other groups and atoms behave as they do cannot be entered into here. Table A1.1 shows the inductive effects for some common groupings.

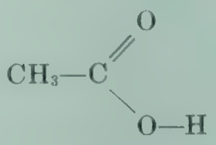
The effects produced by the inductive effect are as follows:

(*a*) *Polarity.* Polarity is important in deciding partitioning behavior between immiscible solvents, and therefore in determining partition coefficients and behavior in chromatograms. Polar molecules are either ionic or electronically lopsided. If one-half of a molecule is more negative than the other, the molecule is polar. Of course, ionized organic material is very polar, but also $CH_3—CH_2 \rightarrow Cl$ is polar, whereas $CH_3—CH_3$ is not, nor is $Cl \leftarrow CH_2—CH_2 \rightarrow Cl$, for here there is a symmetry of electronic shifts. The basic rule in partitioning between solvents is "like attracts like." Since water is very polar due to the asymmetry:

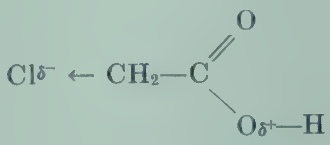


it follows that polar compounds will prefer water to organic solvents, which tend to be apolar. This also explains why on a reverse phase paper chromatogram, where the paper is the nonpolar phase, phosphorothionates will move less readily on the paper than their more polar phosphate analogs, for $P{=}S$ is less polar than $P{=}O$ (Table A1.1).

(*b*) *Acidity and Basicity.* Acids, by definition, release protons (i.e., hydrogen ions) H^+. The more readily they release protons, the stronger acids they are. The acid strength is given by the pK_a, the pH at which 50% of the acid is ionized. Weak acids have a high pK_a: the environment has to be very deficient in protons (high pH) before such acids will release many protons. If we consider acetic and chloroacetic acids, we note that the chlorine of the chloroacetic acid has made the hydroxyl oxygen more electrophilic, by an inductive effect. Consequently, the oxygen binds the proton less excellently, and the proton escapes more easily, so that chloroacetic acid is a stronger acid than acetic acid.*



Acetic acid
pK_a 4.7



Chloroacetic acid
pK_a 2.9

But as the chlorine is moved further away from the carboxyl group, its influence rapidly decreases: one extra CH_2 group interposed (chloropropionic acid) gives a pK_a of 4.1, two extra groups (chlorobutyric acid) give a pK_a of 4.5.

Basicity can be defined as proton-binding capacity. A low pK_a in this case means a weak base, for it implies that a high proton concentration (low pH) is necessary before the base can be induced to bind many protons. Electrophilic groups close to the binding site reduce the negative character of the site and, thus, weaken proton-binding capacity and hence the basicity. Thus, the electrophilic hydroxyl group is base-weakening:

NH_3	$NH_2 \rightarrow OH$
Ammonia	Hydroxylamine
pK_a 9.3	pK_a 6.0

(*c*) *Reaction Rates.* Let us consider any one class reaction, such as hydrolysis of organophosphates. The rate of the reaction is entirely controlled by three factors: the nature of the organophosphate (the "reactant"), the nature of the reagent (OH^-, H^+, or H_2O), and the environment (solvent, temperature, etc.). Now let us consider a series of reactants, and ask why with one particular reagent and in one particular environment, the extent of the reaction differs. For instance, why do various organophosphates differ in alkaline hydrolyzability? Why do organophosphates differ in the ability to inhibit cholinesterase?

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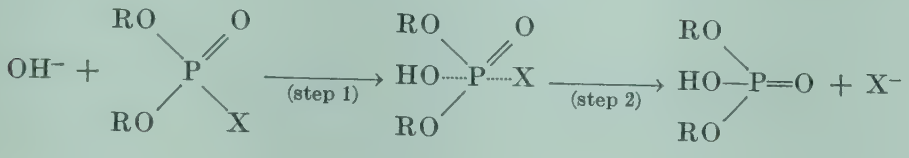
* In the formulae, the important electrophilic effect of the carbonyl oxygen upon the —OH has been neglected for simplicity, since it is common to both molecules.

Reactions involving a reagent can be considered in two parts: in the first step the reagent has to approach the reaction site and (usually) combine with it. In the second step some rearrangement may occur. In fact, these two steps may occur concurrently, as discussed for phosphate hydrolysis on page 30, so that in the hydrolysis

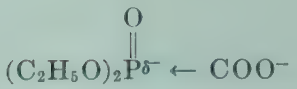


(I)

the "intermediate" (I) has, in most cases, no finite existence. Even in these cases, one may discuss the factors influencing (step 1), realizing that the factors that promote (step 1) are likely to affect (step 2) differently. Now the reactions of importance in this book all concern organophosphates, and consist of: their hydrolysis, their reaction with enzymes, and the spontaneous and induced hydrolysis of the phosphorylated enzyme. In these cases it seems that (step 1) is usually the most critical, i.e., is rate-controlling. Now, let us consider the factors which influence it.

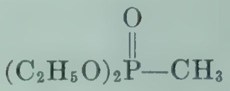
As long as we are considering only differences in the nature of the reactant, only two factors are important: steric and electronic. The steric factors are the spatial, geometric aspects which decide the effectiveness with which the reacting center in the reactant is exposed to the reagent. In organophosphates, the reacting center is commonly the phosphorus, as far as the toxicologically interesting reactions are involved. The electronic factors are those which control the effectiveness of the reaction when the reacting center meets the reagent.

Reactions are of two kinds. (*a*) Those in which the reagent attacks a negative site; the reagent is then said to be electrophilic. This is the case when a phosphate (the reagent) phosphorylates the esteratic site of cholinesterase (the reactant). (*b*) Those in which the site attacked is positive. The reagent is then nucleophilic. An example is an attack on the phosphorus (now a reactant) by the reagent OH^- in alkaline hydrolysis. The terms "negative" and "positive" indicate not only fully ionized sites but more often those bearing only a formal charge, or in other cases those which express their intrinsic electron-displacing capacity (as shown in Table A1.1) which may or may not be modified by neighboring groups. The prevailing rule is simply that opposites attract, and the more opposite the more the attraction. We would, therefore, expect alkaline hydrolyzability to increase in a series of organophosphates whose phosphorus is rendered progressively more positive by varying its substituents:



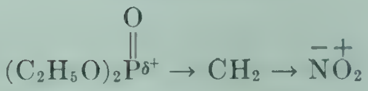
$$(C_2H_5O)_2\overset{\overset{O}{\|}}{P}{}^{\delta-} \leftarrow COO^-$$

Difficult to attack by OH⁻



$$(C_2H_5O)_2\overset{\overset{O}{\|}}{P}-CH_3$$

Intermediate



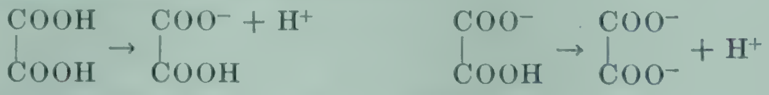
$$(C_2H_5O)_2\overset{\overset{O}{\|}}{P}{}^{\delta+} \rightarrow CH_2 \rightarrow \overset{-\,+}{NO_2}$$

Easily attacked by OH⁻

The Field Effect

We have seen how the inductive effect fades away as the number of atoms through which it has to be transmitted is increased. However, if the chain of atoms involved is long enough and flexible enough, the electron-affecting group may be brought close to the active center and produce an effect transmitted not via the chain but via the solvent. This is the field effect.

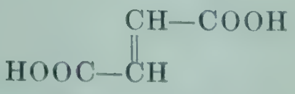
Consider, for instance, a dicarboxylic acid: the removal of the first H^+ leaves a negative carboxylate ion which greatly reduces the tendency of the second H^+ to be removed. This is seen in the fact that the second pK_a is far less than the first, e.g., oxalic acid:



$$\begin{matrix}COOH\\|\\COOH\end{matrix} \rightarrow \begin{matrix}COO^- + H^+\\|\\COOH\end{matrix} \qquad \begin{matrix}COO^-\\|\\COOH\end{matrix} \rightarrow \begin{matrix}COO^-\\|\\COO^-\end{matrix} + H^+$$

First ionization, $pK_a = 1.23$ Second ionization, $pK_a = 4.19$

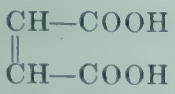
We anticipate that in dicarboxylic acid with its carboxyls more separated, the difference in pK_a values will be less. But with maleic acid and fumaric acid, which have identical numbers of atoms interposed between the carboxyls, we find that the pK_a values are quite different, and that the difference between the first and second pK_a is far less for fumaric than for maleic acid.



$$\begin{matrix} & CH-COOH\\ & \|\\ HOOC- & CH\end{matrix}$$

Fumaric acid

$$pK_a: \left.\begin{matrix}\text{first} = 3.0\\ \text{second} = 4.4\end{matrix}\right\}\begin{matrix}\text{difference}\\ 1.4\end{matrix}$$



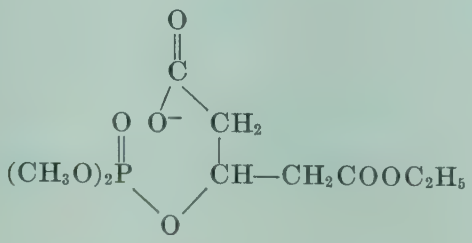
$$\begin{matrix}CH-COOH\\ \|\\ CH-COOH\end{matrix}$$

Maleic acid

$$pK_a: \left.\begin{matrix}\text{first} = 1.9\\ \text{second} = 6.2\end{matrix}\right\}\begin{matrix}\text{difference}\\ 4.3\end{matrix}$$

Since maleic acid differs only geometrically from fumaric acid, the different behaviors are due to space-transmitted (or rather solvent-transmitted) effects, not to effects transmitted through the atomic chain.

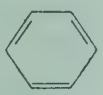
In malathion-β-monoacid, the carboxylate ion is too far from the phosphorus to have an inductive effect; but the ion can approach the phosphorus and have a field effect.

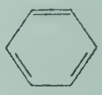


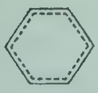
The Mesomeric Effect

When one has a conjugated system, i.e., a system of alternate single and double bonds as in aromatic compounds, the mesomeric effect may occur. It is important because (as we shall see) it may actually reverse the result expected were a simple inductive effect to be involved.

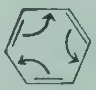
The underlying principle is: if for a given molecule or ion one may draw different possible structures which all have their nuclei in the same position and all have the same number of paired electrons, the actual structure of the molecule or ion is a form intermediate between these possible structures. This phenomenon is known as resonance or mesomerism. The best known case is for benzene, which exists in neither of the two possible forms (I) or (II), but instead in the intermediate form (III), where one has six "one and a half" bonds:


(I)


(II)

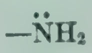

(III)

In order to show that form (I) is an unreal formulation, one can indicate the shift of electrons towards form (III) as follows:



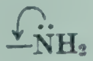
This helps to remind one that a bond is an electron pair, and that a shift in electrons is, thus, a shift in bonding.

Now, let us consider how this influences electrophilic and nucleophilic effects. Table A1.1 showed that —NH_2 has a weak electrophilic effect. But let us consider what happens when the amino group is attached to a phenol molecule. The nitrogen has an unshared electron pair which can tend to go towards making a single bond into a double bond, leaving a charge on the nitrogen, i.e., (I) can go via (II) to (III).



$-\ddot{N}H_2$

(I)

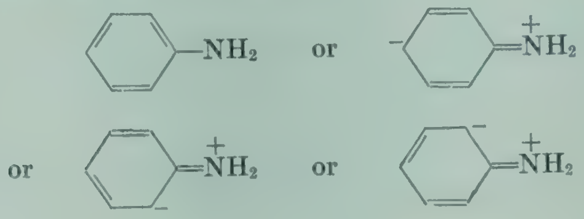


(II)

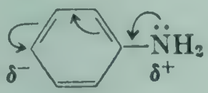
$=\overset{+}{N}H_2$

(III)

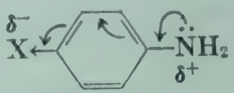
Thus, aniline can be drawn either as:



In fact, aniline will exist as some intermediate form, but this intermediate form will have a partial negative charge on the para and ortho positions; we may describe the way the partial charge on the para position is formed by:



If a substituent is now attached to the *p*-position, it will be subject to the inductive effect of the δ^- on that position. Summing up, and comparing the situation with the aliphatic NH_2 situation:



$\overset{\delta+}{X}\rightarrow CH_2\rightarrow\overset{\delta-}{N}H_2$

We see that aminophenol has a nucleophilic effect, even though aminomethyl has a weak electrophilic effect.

Table A1.2 gives a list of the mesomeric effects of common groupings.

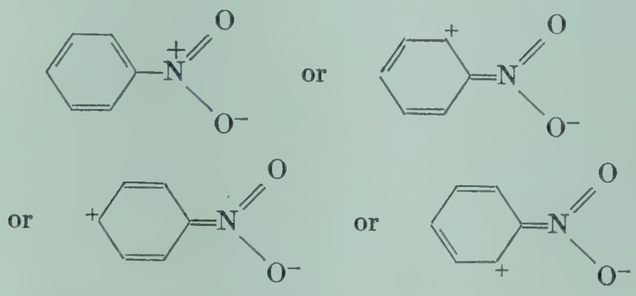
TABLE A1.2. MESOMERIC EFFECTS[a]

Mesomeric effects	Reaction
Nucleophilic:	$-O^- > -OR > -SR$ $-NR_2 > -OR > -F > -Cl > -Br > -I$
Electrophilic:	$=\overset{+}{N}R_2 > =NR$ $=O > =NR > =CR_2$ $\equiv N > \equiv CR$ $=S > =O$

[a] *Note:* A more correct usage is to call the nucleophilic mesomeric effect "the +M effect" and the electrophilic mesomeric effect "the −M effect," cf. Table A1.1.

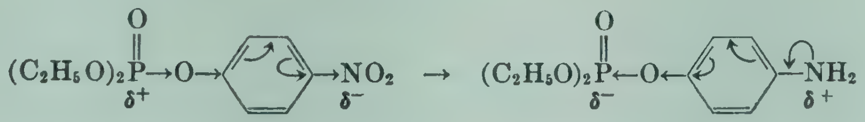
It will be noted that several groups behave as NH_2 in that they have a nucleophilic mesomeric action in spite of an electrophilic inductive action.

When a group with an inductive effect but no mesomeric effect is attached to a conjugated system such as benzene, one also finds the phenomenon of its influence being mostly on the ortho and para carbons of the benzene. In such cases, an electrophilic inductive substituent always makes those carbons electrophilic, and a nucleophilic inductive substituent makes them nucleophilic. For example, the nitro group has a strong electrophilic inductive effect. If we now attach it to phenol, we get the following possibilities:



Consequently, the actual form is an intermediate one, with a δ^+ on the ortho and para carbons.

If we compare the *p*-nitrophenyl group with the *p*-aminophenyl group, we see that the first group will have an electrophilic inductive effect on its substituents, the second will have a nucleophilic effect. Consequently, the reduction of paraoxon to aminoparaoxon



produces a marked change in the electronic nature of the phosphorus. And indeed it is known (page 233) that this reduction greatly reduces both the anticholinesterase activity and the toxicity of the compound.

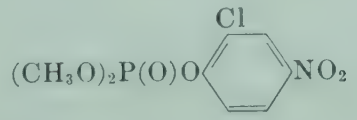
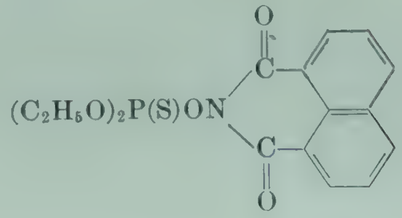
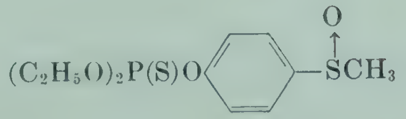
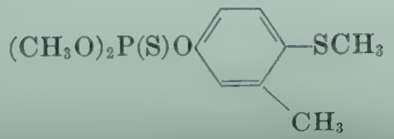
It is important to note from the four possible aniline and nitrophenol structures given above, that only para and ortho substituents are markedly affected, for only these positions bear a δ. Consequently, one predicts that reduction of the *m*-nitrophenyl analog of paraoxon would result in a less drastic alteration in activity.

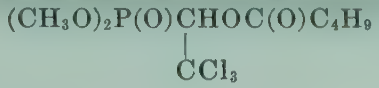
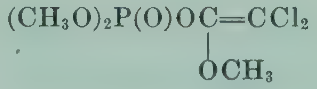
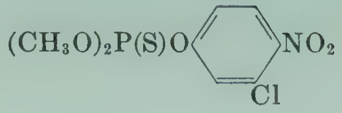
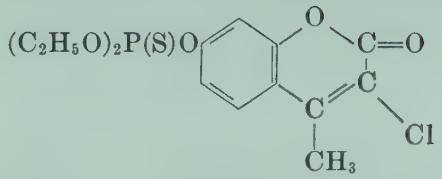
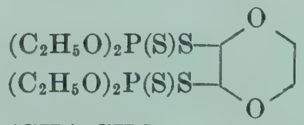
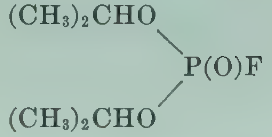
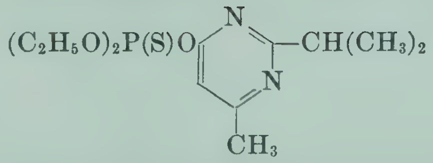
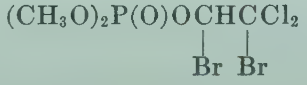
APPENDIX 2

Structure of Organophosphates

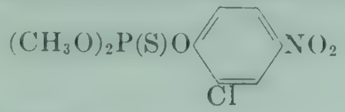
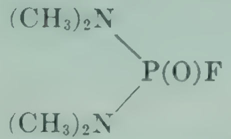
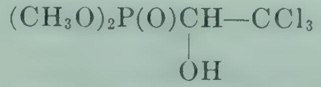
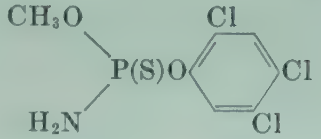
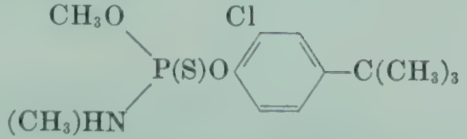
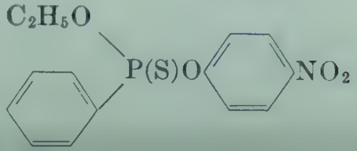
APPENDIX 2

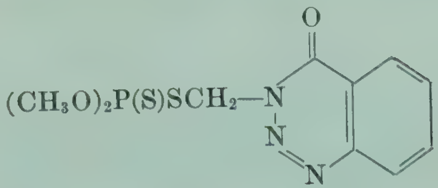
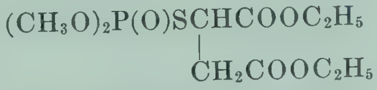
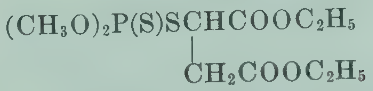
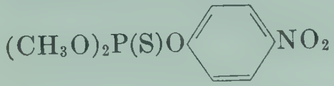
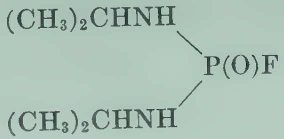
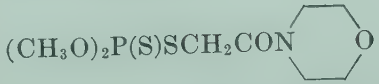
NOTE: *Names beginning with capital letters are trade names; with small letters, common names. Not all the compounds listed are mentioned elsewhere in this book. (See footnote on page 399 for references.)*

Name used in text	Other names	Manufacturer	Structure
acethion	—	—	$(C_2H_5O)_2P(S)SCH_2COOC_2H_5$
acethion amide	—	—	$(C_2H_5O)_2P(S)SCH_2CONH_2$
acetoxon	—	—	$(C_2H_5O)_2P(O)SCH_2COOC_2H_5$
Amiton	—	I.C.I., Chipman	$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$
American Cyanamid 4138	—	American Cyanamid	
American Cyanamid 12008	—	American Cyanamid	$(C_2H_5O)_2P(S)SCH_2SCH(CH_3)_2$
Bayer 22408	—	Bayer	
Bayer 25141	—	Bayer	
Baytex	Bayer 29493	Bayer	

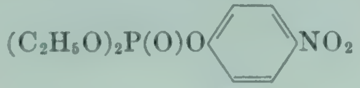
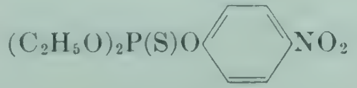
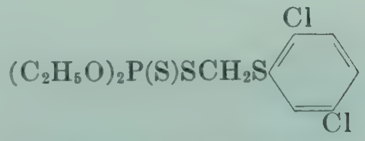
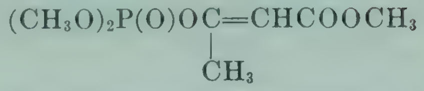
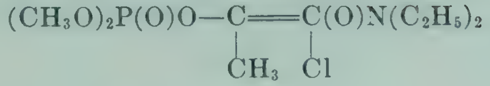
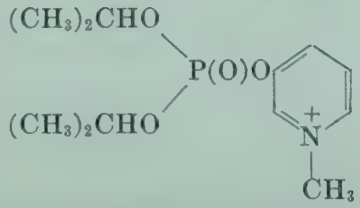
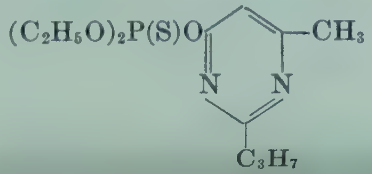
butonate	—	—	 $(CH_3O)_2P(O)\underset{\displaystyle CCl_3}{\underset{\mid}{CH}}OC(O)C_4H_9$
Chlorophan	G27044	Bayer	 $(CH_3O)_2P(O)O\underset{\displaystyle OCH_3}{\underset{\mid}{C}}{=}CCl_2$
Chlorthion	—	Bayer, Chemagro	
Co-Ral	Resitox Muscatox Asuntol	Bayer, Chemagro	
DDVP	—	Montrose	$(CH_3O)_2P(O)OCH{=}CCl_2$
Delnav	Hercules AC528	Hercules	
DFP	—	—	
Diazinon	—	Geigy	
Dibrom	—	California Spray	 $(CH_3O)_2P(O)O\underset{\displaystyle Br}{\underset{\mid}{CH}}\underset{\displaystyle Br}{\underset{\mid}{C}}Cl_2$

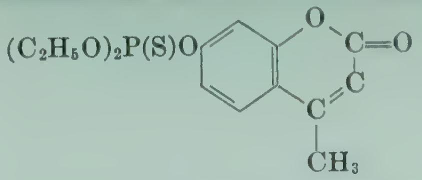
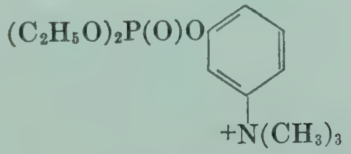
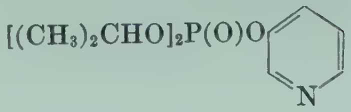
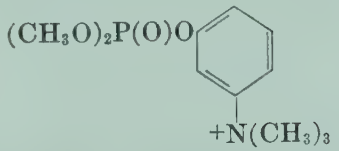
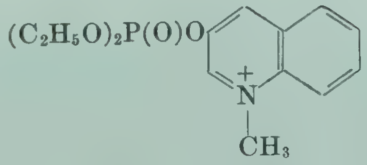
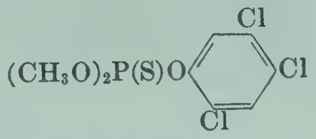
Appendix 2 (*Continued*)

Name used in test	Other names	Manufacturer	Structure
dicapthon	American Cyanamid 4124	American Cyanamid	
dimefox	Hanane	Fisons Pest Control	 $((CH_3)_2N)_2P(O)F$
dimethoate	CL12880 Rogor	American Cyanamid, Montecatini	$(CH_3O)_2P(S)SCH_2C(O)NHCH_3$
Dipterex	Bayer L13/59 Dylox	Bayer, Chemagro	 $(CH_3O)_2P(O)CH(OH)—CCl_3$
Disyston	Bayer 19639 dithio-Systox	Bayer, Chemagro	$(C_2H_5O)_2P(S)SCH_2CH_2SC_2H_5$
Dow ET-15	—	Dow	
Dowco 109	Narlene	Dow	
Ekatin	—	Sandoz	$(CH_3O)_2P(S)SCH_2CH_2SC_2H_5$
EPN	—	DuPont	

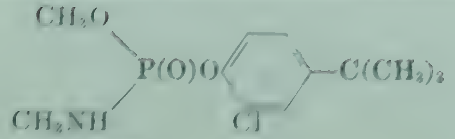
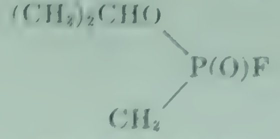
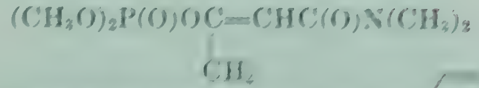
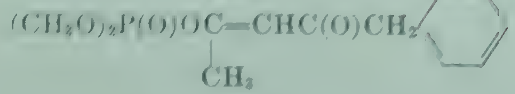
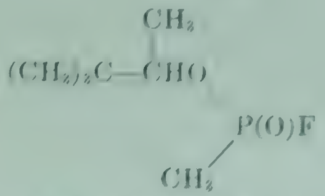
Ethion	Nialate Niagara 1240	Niagara	$(C_2H_5O)_2P(S)SCH_2SP(S)(OC_2H_5)_2$
Guthion	Gusathion	Bayer, Chemagro	
HETP	—	Monsanto	A preparation containing TEPP as active ingredient
iso-OMPA	—	—	$[(CH_3)_2CHNH]_2P(O)OP(O)[NHCH(CH_3)_2]_2$
ketothion	—	—	$(C_2H_5O)_2P(S)SCH_2C(O)CH_3$
malaoxon	—	—	 $(CH_3O)_2P(O)S\underset{\substack{\vert \\ CH_2COOC_2H_5}}{C}HCOOC_2H_5$
malathion	—	American Cyanamid	 $(CH_3O)_2P(S)S\underset{\substack{\vert \\ CH_2COOC_2H_5}}{C}HCOOC_2H_5$
meta-Systox	methyl Systox methyl demeton	Bayer, Chemagro	$(CH_3O)_2P(S)OC_2H_4SC_2H_5$ (thiono isomer) $(CH_3O)_2P(O)SC_2H_4SC_2H_5$ (thiolo isomer)
methyl parathion	E601	American Cyanamid, Chemagro, Monsanto, Victor	
mipafox	Isopestox	Fison's	 $\begin{array}{l}(CH_3)_2CHNH\diagdown \\ \qquad\qquad\quad P(O)F \\ (CH_3)_2CHNH\diagup\end{array}$
Morphothion	—	Sandoz	
NPD	—	DuPont	$(C_3H_7O)_2P(S)OP(S)(OC_3H_7)_2$

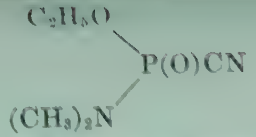
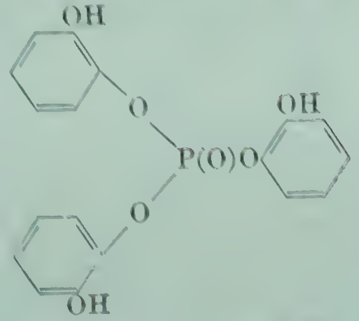
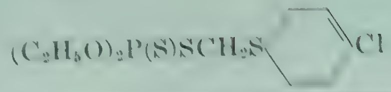
Appendix 2 (*Continued*)

Name used in text	Other names	Manufacturer	Structure
OS1836	—	Shell	$(CH_3O)_2P(O)OCH{=}CHCl$
paraoxon	E600	Bayer	
parathion	E605 Thiophos	American Cyanamid, Bayer, Chemagro, Monsanto	
Phenkaptone	—	Geigy	
Phosdrin	Shell 2046	Shell	 $(CH_3O)_2P(O)O\underset{CH_3}{\underset{\vert}{C}}{=}CHCOOCH_3$
phosphamidon	—	Ciba	 $(CH_3O)_2P(O)O{-}\underset{CH_3}{\underset{\vert}{C}}{=}\underset{Cl}{\underset{\vert}{C}}(O)N(C_2H_5)_2$
phosphopyristigmine	—	—	
Phostex	—	Niagara	$(RO)_2P(S)SSP(S)(OR)_2$ where R = mixed ethyl and isopropyl
Pirazinon	—	Geigy	

Potasan	E838	Bayer	
propoxon	—	—	$(C_2H_5O)_2P(O)SCH_2CH_2COOC_2H_5$
prothion	—	—	$(C_2H_5O)_2P(S)SCH_2CH_2COOC_2H_5$
Ro 30340	—	Roche	
Ro 30351	—	Roche	
Ro 30412	—	Roche	
Ro 30422	—	Roche	
ronnel	Dow ET14 (tech.), Dow ET57 (pure), Trolene, Korlan, Viozene	Dow	

Appendix 2 (*Continued*)

Name used in text	Other names	Manufacturer	Structure
Ruelene	M1261	Dow	
sarin	GB	—	
schradan	—	Fison's, Monsanto, Murphy	$[(CH_3)_2N]_2P(O)OP(O)[N(CH_3)_2]_2$
Shell 3562	—	Shell	 $(CH_3O)_2P(O)O\underset{CH_3}{\underset{\vert}{C}}{=}CHC(O)N(CH_3)_2$
Shell 5539	—	Shell	
soman	—	—	
sulfotepp	—	Monsanto, Victor	$(C_2H_5O)_2P(S)OP(S)(OC_2H_5)_2$
Systox	demeton	Bayer, Chemagro	$(C_2H_5O)_2P(S)OC_2H_4SC_2H_5$ (thiono isomer) $(C_2H_5O)_2P(O)SC_2H_4SC_2H_5$ (thiolo isomer)

tabun	—	—	 $(C_2H_5O)(CH_3)_2N\text{P(O)CN}$
TEPP	—	Monsanto, Victor	$(C_2H_5O)_2P(O)OP(O)(OC_2H_5)_2$
Tetram	—	I.C.I., Chipman	Oxalate salt of Amiton, q.v.
Thimet	phorate	American Cyanamid	$(C_2H_5O)_2P(S)SCH_2SC_2H_5$
TOCP	TCP TOTP	Stauffer	
Trithion	—	—	

Note: Extensive references and formulae for these and less common organophosphates may be found in: Holmstedt, B., *Pharmacol. Rev.* **11**, 567 (1959); Kenaga, E. E., *Bull. Entomol. Soc. Am.* **6**, 53 (1960); Martin, H., "Guide to the Chemicals Used in Crop Protection," 3rd ed. Canada Dept. of Agriculture, Ottawa, 1957; and Negherbon, W. O., "Handbook of Toxicology," Vol. 3. Saunders, Philadelphia, 1959.

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Numbers in parentheses are reference numbers and are included to assist in locating the reference where the authors' names are not mentioned in the text. Numbers in italics refer to the page which the reference is listed.

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